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Relationships Between Pteridines and Other Heterocycles (Purines, Riboflavin, and Vitamin B_{12})

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THIS DISCUSSION will deal primarily with reaction schemes derived from experiments done in our laboratories with protozoa. Pathways worked out with other organisms or systems will serve only for supplementary information and, because of limitations of space, will not be discussed in detail.

Several years ago it was noted that *Crithidia fasciculata*, a protozoan parasite of mosquitoes, required inordinately high levels of a conjugated pteridine,‡ e.g., folic acid, for growth. The lower efficiency of leucovorin than of folic acid in *C. fasciculata*[2] further emphasized that the role of conjugated pteridines in this organism differed from the usual microbial pattern, i.e., folic acid → leucovorin → thymine.

A folic acid-sparing action of culture supernatants of microorganisms, e.g., *Ochromonas malhamensis*,[1,2] and of tissue extracts, e.g., liver,[1,2] could not be explained by the conjugated pteridine content of the preparations. Indeed, the folic-sparing action was retained even after the preparations were hydrolyzed so as to destroy the conjugated pteridines.[2]

The "Crithidia factor" content of these natural materials was too low for concentration and identification of the active fraction. Fortunately most commercially available "purified" purine ribosides were contaminated with Crithidia factor. Guanosine was especially rich,[2] and the active fraction was the mother liquor obtained during recrystallization of this riboside. Consistent contamination of purines with unconjugated pteridines had been noted by others.[3] Comparison of the properties, distribution, and ability of conjugated pteridines to serve as precursors[2] all indicated that Crithidia factor might be an unconjugated pteridine with the following substituents: 2-amino, 4-hydroxy, and 6-R, with R an aliphatic chain bearing at least one hydroxyl group.[2] This possibility was soon confirmed; the full structure of the Crithidia factor in human urine was found to be 2-amino, 4-hydroxy, 6-dihydroxypropyl-pteridine, and it was given the trivial

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‡ Conjugated pteridines: the pteridine nucleus with some aromatic substituent at carbon 6, usually *p*-aminobenzoic acid, or *p*-aminobenzoylglutamic acid. Unconjugated pteridines: the pteridine nucleus with an aliphatic substituent at carbon 6 or carbon 7.

name biopterin.[4] We found biopterin chromatographically identical with the Crithidia factor in *O. malhamensis*,[2,5] in liver,[2,5] and in the guanosine impurity.[2] Several unconjugated pteridines may serve as sources of Crithidia factor.[4–7] The qualifications are that the 6-substituent be either hydroxymethyl or a longer aliphatic chain with two vicinal hydroxyl groups.

Now the seemingly anomalous role of folic acid in the metabolism of *C. fasciculata* could be clarified.[6] As shown in Table I, folic acid (or leucovorin) when serving as sole exogenous pteridine has two functions: (a) the usual one-carbon role which results in the production of thymine (and later of DNA); (b) the precursor of an independently functioning unconjugated pteridine called Crithidia factor. It should be noted that leucovorin, a poor source of Crithidia factor, is, in the presence of preformed Crithidia factor, at least as efficient as folic acid in one-carbon metabolism. Thus the high leucovorin requirement, when it serves as sole pteridine source, can be explained by the very limited ability of this compound to serve as a precursor for Crithidia factor.

TABLE I

Dissection of Pteridine Functions in *Crithidia fasciculata*

Pteridine function satisfied by	Concentration required for full growth	Metabolic function: synthesis of
Folic acid	0.06–0.1 mg %	Thymine and Crithidia factor
Leucovorin	0.3 –0.6 mg %	Thymine and Crithidia factor
Folic acid plus Crithidia factor[a]	0.01–0.1 μg %	Thymine
	0.01–0.1 μg %	See text
Leucovorin plus Crithidia factor[a]	0.01–0.1 μg %	Thymine
	0.01–0.1 μg %	See text
Thymine (or thymidine) plus Crithidia factor[a]	1.0 –3.0 mg %	Nucleic acids
	0.01–0.1 μg %	See text
Folic acid (or leucovorin) plus 2,4,5-Triamino, 6-hydroxypyrimidine and Sugar[b]	0.01–0.1 μg %	Thymine
	0.5 mg %	
	0.1 –1.0 mg %	Crithidia factor

[a] In these and subsequent experiments 2-amino, 4-hydroxy, 6-trihydroxypropylpteridine was used.

[b] For example, glucose. Also see Table III.

For all the heterocycles to be discussed we will consider: (a) their biosynthesis from pool reactions and (b) *de novo* synthesis. Both routes may be active in the same organism.

SYNTHESIS OF CRITHIDIA FACTOR FROM FOLIC ACID

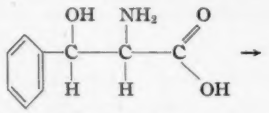
There is little direct evidence on the mechanism of the splitting of conjugated pteridines in protozoa. Recent studies on the cleavage of folic acid by enzymes in red blood cells[8,9] and chick peas[9] suggest a model reaction. The fact that aminopterin* can also serve as substrate for this enzyme[8,9] and that aminopterin is a fair source of total pteridine for *C. fasciculata*[1] supports this view. It is tempting to think of the reaction in protozoa as proceeding: folic acid → some tetrahydrofolic acid derivative → *p*-aminobenzoylglutamic acid + some stripped pteridine, which might be 2-amino-, 4-hydroxytetrahydro-pteridine itself; the 6-formyl, or the 6-hydroxymethyl derivative; the latter compound in oxidized form does have Crithidia factor activity.[6] The source of the rest of the chain at the 6-position will no doubt soon be elucidated with isotope experiments showing the incorporation of a fragment from a compound which can donate a hydroxymethyl group. It is now conceded that a "cofactor form" of folic acid transfers hydroxymethyl groups,[10] but no studies have yet appeared on the length of the hydroxylated aliphatic chain which may be transferred. Our experiments indicate that if the building of the 6-R substituent of the Crithidia factor series includes addition of hydroxymethyl or hydroxyethyl, some "cofactor" folic acid is involved. Thus we have another indication of a compound which may serve as substrate and as cofactor in the same reaction.

The reverse reaction, Crithidia factor → folic acid, which in *C. fasciculata* either does not occur or does so only negligibly,† may be con-

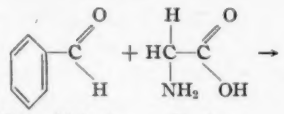
* The possibility that this reaction may be triggered by an impurity consisting of folic acid cannot be excluded at this time.

† The direction of the pteridine interconversions is probably pH-dependent. Conjugated pteridine biosynthesis by proliferating cells of *C. fasciculata* may be impossible to demonstrate because the organism will not grow at pH below 7. It may, however, be possible to demonstrate the reaction using resting cells.

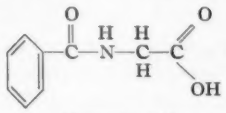
sidered analogous to the cleavage of L-threo-β-phenylserine and L-erythro-β-phenylserine.[11] The latter reaction is thought to proceed:



β-phenylserine



Benzaldehyde Glycine



Hippuric acid

and requires pyridoxal phosphate.[11]

At the termination of this reaction a peptide link is established. The parallel pteridine reaction may indeed be the one diagrammed in an earlier paper[6] in which a 6-hydroxymethyl pteridine was suggested as an intermediate.

That vitamin B_6 or some derivative is involved in the reaction, folic acid→Crithidia factor, is one interpretation of the experiment shown in Table II. In this experiment we attempted to bypass the vitamin B_6 requirement of *C. fasciculata* by supplying supposed products of vitamin B_6 metabolism (a discussion of the bypass technic is given elsewhere in this symposium[12]). We found that pyridoxamine is spared by a mixture of amino acids when preformed Crithidia factor is present (see Table II, second part) but not when Crithidia factor must be manufactured from folic acid (see Table II, first part). Thus vitamin B_6 is a cofactor in the reaction folic acid → Crithidia factor.

TABLE II

Partial Replacement of the Vitamin B_6 Requirement of *Crithidia fasciculata* in the Presence of Unconjugated Pteridines

Additions to pteridine assay medium[a] lacking vitamin B_6	Supplements	
	None	Amino acids[b]
Folic acid (0.1 mg %) plus Pyridoxamine·2HCl		
0	0	0
0.01 μg %	0.15[c]	0.26
0.03	0.27	0.31
0.10	0.54	0.71
0.30	0.64	0.80
1.0	1.21	0.95
3.0	1.24	1.27
Folic acid (0.3 μg %) and 2-NH_2, 4-OH, 6-trihydroxypropylpteridine (10 μg %) plus Pyridoxamine·2HCl		
0	0	0.22
0.01	0.18	0.50
0.03	0.26	0.65
0.10	0.87	1.10
0.30	0.59	1.14
1.0	1.18	1.26
3.0	1.20	1.30

[a] See Nathan and Cowperthwaite[27] for formula.

[b] DL-Alanine, 0.05%; DL-asparagine·H_2O, 0.1%; L-glutamic acid, 0.1%; L-tyrosine, 0.01%; glycine, 0.05%.

[c] Growth in optical density units as measured by a Welch Densichron.

DIRECT SYNTHESIS OF CRITHIDIA FACTOR

The chemist synthesizes unconjugated pteridines by condensing 2,4,5-triamino, 6-hydroxypyrimidine with a compound which will close the pyrazine ring and also supply the desired side chain. The yield is usually small (less than 2 per cent; see, for example, Patterson *et al.*[4]). Because of the difficulty of directing the side chain to either the 6 or 7 position, a mixture of 6 and 7 substituted compounds is obtained, although one position may be favored by the proper choice of catalysts. Once the desired compound is obtained, the yield is further reduced by purification. In one recent synthetic method[13] an 86 per cent yield of the 6-L-erythro-trihydroxypropyl derivative is claimed, but other workers have found this difficult to repeat.[14]

To understand better the biosynthesis and functions of unconjugated pteridines one can supply raw materials to an organism, e.g., *C. fasciculata*, which can condense a substituted pyrimidine with an appropriate side chain and use the resultant pteridine for some readily detectable process, such as growth.

When *C. fasciculata* was grown in a defined medium with sorbitol as chief substrate (Table II of Nathan *et al.*[5]), the usual dual pteridine growth requirements were demonstrable, i.e., a conjugated and an unconjugated pteridine (i.e., some source of Crithidia factor) were required (Table III). In the presence of only enough folic acid to satisfy catalytic functions and in the absence of preformed Crithidia fac-

tor, 2,4,5-triamino, 6-hydroxypyrimidine plus a compound which could close the pyrazine ring and supply the appropriate side chain satisfied the Crithidia factor requirement (Table III). This result implies that a synthetic block in *C. fasciculata*, manifested by a requirement for unconjugated pteridines, is at least one step before the completion of pteridine rings and that pteridine biosynthesis parallels chemical synthesis at least in the terminal stages.

When amount of Crithidia factor synthesized was judged by the amount of growth of *C. fasciculata*, thymine or thymidine did not permit high yields of the factor. Indeed, high yields were consistently obtained only in the presence of small amounts of folic acid (see Table III); the possibility of a folic acid-catalyzed step in this reaction should not be excluded.

There are at least two interpretations for the inactivity of some sugars (see Table III) in ring closure: (a) they are not used in this biosynthetic reaction; or (b) they are used but the resulting pteridines are lethal to *C. fasciculata*. What really happens awaits experiments with resting cells from which the biosynthetic products can be isolated and identified.

TABLE III

Biosynthesis of Unconjugated Pteridines by *Crithidia fasciculata*

Additions per 100 ml of medium[a]		Supplements per 100 ml: None	Supplements per 100 ml: Folic acid (0.5 μg)
None		0.08[c]	0.07
2-NH_2, 4-OH, 6-trihydroxypropyl pteridine	0.5 μg	0.08	1.07
2,4,5-Triamino, 6-hydroxypyrimidine (0.5 mg)[b] plus			
No additions	..	0.06	0.11
D-Xylose	0.1 mg	0.05	0.37
	1.0	0.06	0.12
D-Arabinose	0.1	0.08	0.46
	1.0	0.10	0.40
D-Ribose	0.1	0.11	0.18
	1.0	0.09	0.16
Gluconolactone	0.1	0.09	1.23
	1.0	0.08	1.00
D-Galactose	0.1	0.11	1.02
	1.0	0.08	0.86
D-Mannose	0.1	0.13	0.90
	1.0	0.08	1.29
L-Rhamnose	0.1	0.10	1.06
	1.0	0.08	0.62

[a] The sorbitol medium given in Table II in 5 was used.
[b] Results are the same whether this compound is mixed with the base before autoclaving or added aseptically.
[c] Growth as optical density units.

FACTORS AFFECTING THE PRODUCTION OF PTERIDINES BY MICROORGANISMS

Only two organisms will be considered: the alga, *Ochromonas danica*, and the coccoid bacterium, *Gaffkya homari*. Early experiments with *O. malhamensis* showed that both Crithidia factor and a compound with chromatographic characteristics of Citrovorum factor were formed during growth. Chromatographic studies not reported here indicated that the pteridines produced by *O. malhamensis* and *O. danica* are identical.

The following question arose: How do different stressors affect the kind and quantity of pteridines produced? The choice of stressors presented a problem because chemical agents that might interfere with pteridine production in *Ochromonas* were toxic to the pteridine assay organism, *C. fasciculata*. Heat is a convenient stressor which can be carefully controlled, and results obtained through its use might point to parallel problems in animals, e.g., thyrotoxicosis, fever, and some types of radiation damage (see discussion by Hutner *et al.*[16]).

For algal heat stress, *O. danica* was grown at 30°C (summer room temperature in our laboratories) or at 36°C. The growth medium lacked vitamin B_{12} but contained a carbohy-

TABLE IV

Effect of Temperature on the Production of Pteridines by *Ochromonas danica*

Pteridine assay medium containing		Supplements: None	Supplements: Folic acid (0.1 μg %)	Supplements: 2-NH_2,4-OH,6-trihydroxypropyl pteridine (0.06 μg %)
No additions		0.05[b]	0.05	0.12
Supernat. of *O. danica* cultures grown at				
30°C	0.003 ml %	0.09	0.81	0.75
	0.01	0.13	1.22	0.90
30°C and hydrolyzed[a]	0.003	0.06	1.10	0.10
	0.01	0.20	1.11	0.08
36°C	0.003	0.08	1.13	0.27
	0.01	0.14	1.23	0.67
	0.1	1.18	1.24	1.26
36°C and hydrolyzed	0.003	0.06	1.07	0.08
	0.01	0.07	1.30	0.10
	0.1	0.10	1.23	0.10

[a] See text and also Reference 5.
[b] Growth as optical density units.

drate, amino acids, metals, thiamine, and biotin. Differential assays (this procedure has been fully discussed elsewhere[5]) of culture supernatants of *O. danica* (Table IV) revealed that (a) the supernatants would satisfy all pteridine requirements of *C. fasciculata;* (b) the hydrolyzed supernatants (only conjugated pteridines were destroyed[2,5]) would satisfy only the Crithidia factor requirement of *C. fasciculata;* (c) heat stress caused a tenfold decrease in total pteridines produced by *O. danica;* and (d) the decrease represented a decrease of conjugated pteridines. The content of unconjugated pteridine did not decrease with the elevation of temperature; indeed, it may actually have increased.

These results point to certain problems, at least with respect to the alga: (a) Do the syntheses of the conjugated and the unconjugated pteridines proceed for a time along a common pathway, and if so, does temperature elevation shift the equilibrium at the point of divergence in favor of unconjugated pteridines? (b) Are all the unconjugated pteridines produced during catabolism of conjugated pteridines, possibly by the route previously detailed, and if so, does 36°C approach the temperature optimum for this catabolic reaction?

The effect of compounds which are intimately involved in pteridine metabolism but themselves lack the pteridine moiety were studied with *G. homari.* This organism has an interchangeable requirement for para-aminobenzoic acid (PABA) or purines.[15] In the experiment shown in Table V, production of conjugated pteridines was below assayable levels. Subsequent experiments showing that conjugated pteridines are produced are considered elsewhere.[15] Table V shows that the production of Crithidia factor varies with the purine (or PABA) supplied to *G. homari.* This organism may therefore constitute a convenient system in which to study, isotopically, conversion of purines to unconjugated pteridines. *G. homari* culture supernatants from hypoxanthine- or guanine-grown organisms were richest in Crithidia factor activity, those from PABA-grown organisms were only half as rich, and those from adenine-grown organisms had only slight activity.

TABLE V

Production of Pteridines by *Gaffkya homari* as Shown by Growth Response of *Crithidia fasciculata*

Additions per 100 ml of pteridine assay medium		Supplements per 100 ml: Folic acid (0.06 μg)	Supplements per 100 ml: 2-NH_2,4-hydroxy-6-trihydroxy propyl pteridine (0.03 μg)
None	—	0	0
Folic acid	0.6 μg	0	0.94[a]
2-NH_2, 4-OH, 6-trihydroxypropyl pteridine	0.03	1.00	0
Supernat. of *G. homari* cult. when growth medium contained			
Adenine	1.0 ml	0.06	0
	3.0	0.21	0
Guanine	0.1	0.04	0
	1.0	0.35	0
	3.0	0.94	0
Hypoxanthine	0.001	0.28	0
	0.01	0.29	0
	0.1	0.49	0
	1.0	0.20	0
	3.0	0.93	0
p-Aminobenzoic acid	1.0	0.18	0
	3.0	0.46	0

[a] Growth as optical density units.

One interpretation of these results (see Table V) is that the hypoxanthine and guanine can be directly involved in pteridine synthesis, possibly with a common substituted 4,5-diaminopyrimidine intermediate. If so, the key substituent on the purine nucleus is 6-hydroxy, which is equivalent to 4-hydroxy of pteridine. Adenine, a 6-aminopurine, either cannot serve as a substrate, or it may be the precursor of a pteridine which is lethal to *C. fasciculata.* Although PABA cannot be a direct precursor of conjugated pteridines, in *G. homari* it could participate in synthesis of conjugated pteridine Crithidia factor probably is then formed from a pool of conjugated pteridines. The process could be similar to the one that occurs in *C. fasciculata* when folic acid is the sole source of preformed pteridine (see Table I) and to that in *O. danica* grown at elevated temperatures.

FUNCTIONS OF PTERIDINE IN CRITHIDIA FASCICULATA

As previously noted[6] conjugated pteridines can have a dual function: the one-carbon role which results in the production of thymine, and the role of substrate in the synthesis of Crithidia

factor from a pteridine pool. We have here presented two possible catalytic roles for conjugated pteridines: (1) in the biosynthesis of Crithidia factor from conjugated pteridine, e.g. folic acid, and (2) in the direct synthesis of unconjugated pteridines from substituted pyrimidines.

We have little direct information on the function of Crithidia factor except that it is involved in reactions vital for growth of *C. fasciculata*. Some clues to the identification of these vital functions may be derived from the observations of others. Isoxanthopterin, an unconjugated pteridine, inhibited the initial oxidation of DOPA but accelerated the production of melanin at the expense of the red intermediates.[17] Enzymatic conversion of phenylalanine to tyrosine requires a pteridine,[18] and the natural cofactor is tentatively identified as an unconjugated pteridine.[19] There are also hormonal functions of pteridines in insects.[20,21] For example, biopterin is the factor in royal jelly that is responsible for the development of the queen bee.[22] A mutant of Drosophila, (rosy^2), which lacks isoxanthopterin, produced many other unconjugated pteridines,[23] some of which have Crithidia factor activity. The absence of the isoxanthopterin in this mutant has recently been tied to the absence of xanthine oxidase.[24] The inhibition of xanthine oxidase (which incidentally has a riboflavin cofactor) by 6-formylpteridine has long been known. One interesting report contains an account of spontaneous "escape" from this inhibition.[25] Could the small amount of growth of *C. fasciculata* that was obtained in the presence of high riboflavin plus Crithidia factor but *no* conjugated pteridine (Table 2 in Nathan *et al.*[6]) reflect a very limited ability of *C. fasciculata* to do the reverse of an analogous reaction?

Another approach is to measure the variation of key products formed in *C. fasciculata* cells and culture supernatants as a function of the several compounds which may be used to satisfy the pteridine requirements. Clinical observations show that in certain diseases which involve vitamin B_{12}, folic acid sometimes relieves symptoms, whereas in other vitamin B_{12}-linked diseases, folic acid may potentiate symptoms. Thus vitamin B_{12} seemed to be the compound to measure in *C. fasciculata* cultures.

Two major types of vitamin B_{12} were measured (Table VI): (a) true vitamin B_{12} by *O. malhamensis* assay, and (b) total vitamin B_{12} (true plus pseudo) by *Euglena gracilis* assay. When folic acid was the sole pteridine source for *C. fasciculata* true vitamin B_{12} was possibly the sole product.* When small quantities of folic acid† and Crithidia factor were fed to *C. fasciculata*, various types of pseudo vitamin B_{12} were produced but apparently not at the expense of true vitamin B_{12}. When thymidine was used to bypass folic acid (in the presence of preformed Crithidia factor) there was a tenfold drop in true vitamin B_{12} without a concomitant drop in pseudo vitamin B_{12}. Indeed, there was a fourfold increase (over the folic acid plus Crithidia factor-grown cultures) in the production of pseudo vitamin B_{12}. The most dramatic change was noted when folic acid† and 2,4,5-triamino, 6-hydroxypyrimidine were fed to *C. fasciculata:* no assayable vitamin B_{12} was produced. Clearly, then, at least one pteridine, an unconjugated one, is needed for any vitamin B_{12} synthesis, and a conjugated pteridine di-

TABLE VI

Production of Vitamin B_{12}-like Materials by *Crithidia fasciculata*

Pteridine functions of *Crithidia* fulfilled by		mμg "vit. B_{12}" per l of whole *Crithidia culture*	
		Euglena assay	Ochromonas assay
Folic acid	0.1 mg %	4.0	4.6
Folic acid plus	0.1 μg %		
2-NH_2,4-OH,6-trihydroxypropyl pteridine	0.1 μg %	6.4	5.2
Thymidine plus	2.0 mg %		
2-NH_2,4-OH,6-trihydroxypropyl pteridine	0.1 μg %	5.2	0.7
Folic acid plus	0.1 μg %		
2,4,5-triamino,6-hydroxypyrimidine plus	0.5 mg %		
Glucose		0	0

* It will be noted that a seemingly impossible situation obtained. There was more true vitamin B_{12} than total vitamin B_{12}! The implications of this result and of similar ones obtained when body fluids are assayed will be discussed fully elsewhere.[28]

† Only enough to satisfy the one-carbon function.

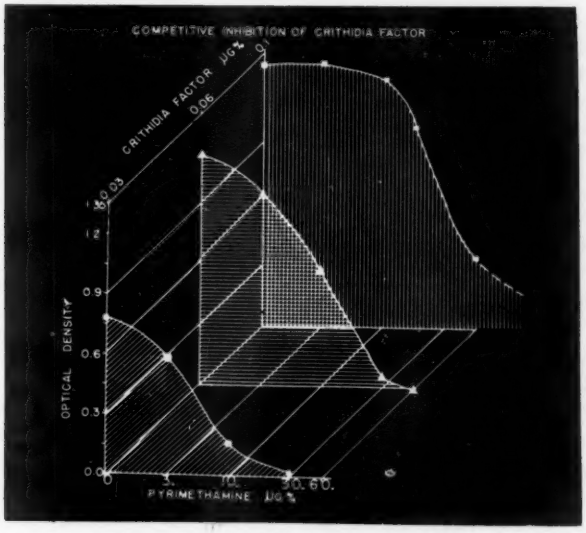


Fig. 1. Annulment by Crithidia factor of pyrimethamine inhibition of growth of *Crithidia fasciculata*.

rects synthesis of the true vitamin B_{12} at the expense of the pseudo.

INHIBITION ANALYSIS AND CRITHIDIA FACTOR

Several years ago it was noted that the "antifolic" activity of diaminopyrimidine antimalarials to *C. fasciculata* paralleled the order of toxicity to plasmodia.[27] To understand better why the most potent drug in this series, pyrimethamine,* is so effective against plasmodia but relatively nontoxic to man (both require folic acid), we studied the pyrimethamine inhibition of pteridines (and their products) in *C. fasciculata*.

We had already shown[27] that pyrimethamine competitively inhibited folic acid and leucovorin and thus ruled out conversion of folic acid to leucovorin as an inhibition locus. In addition to a general focus on pteridine metabolism, we could now dissect this pyrimethamine inhibition. The potency of pyrimethamine rests on its ability to inhibit competitively and simultaneously at more than one site. Three sites will be discussed: conjugated pteridines, unconjugated pteridines, and thymine. Flavins, purines, and vitamin B_{12} will be reserved for future consideration.

* 2,4-Diamino, 5-*p*-chlorophenyl, 6-ethyl pyrimidine.

A graphic illustration of the competitive inhibition of Crithidia factor by pyrimethamine is given in Figure 1. In light of such evidence it was now necessary to establish whether in the presence of preformed Crithidia factor, leucovorin was still competitively inhibited, and if so, what could be the product of the reaction. As shown in the first part of Table VII, inhibition of leucovorin in the presence of Crithidia factor is competitive, whereas inhibition of thy-

TABLE VII

Pyrimethamine Inhibition Patterns in *Crithidia fasciculata*

Additions to pteridine assay medium	Pyrimethamine in µg %			
	None	3.0	10.0	30.0
2-NH_2,4-OH,6-trihydroxypropyl pteridine, 0.03 µg %				
Plus leucovorin				
0.1 µg %	0.75[a]	0.65	0.28	0.08
0.3	0.80	0.70	0.45	0.17
1.0	0.84	0.74	0.62	0.32
Plus thymine				
1.0 mg %	0.88	0.72	0.41	0.14
3.0	0.80	0.69	0.42	0.14
10.0	0.71	0.52	0.43	0.17
Thymine, 1.0 mg %				
Plus 2-NH_2,4-OH,6-trihydroxypropyl pteridine				
0.03 µg %	0.59	0.41	0.13	0
0.06	0.88	0.72	0.41	0.14
0.1	1.06	0.96	0.71	0.23

[a] Growth as optical density units.

mine is noncompetitive. These results confirmed growth studies which showed that (a) either folic acid or leucovorin can enter a reaction series resulting in the formation of a "cofactor" form of conjugated pteridine, and (b) that this "cofactor" functions in thymine synthesis.

The competitive nature of the inhibition of Crithidia factor is not dependent upon how the one-carbon metabolism requirement is satisfied (see Fig. 1 and second part of Table VII). Also, we might be able to identify the product of Crithidia factor metabolism by experiments like that described in Table VII. So far we have few clues about the substrate upon which Crithidia factor acts. Compounds which competitively inhibit Crithidia factor but are inert to conjugated pteridines may aid in identification of the substrate. One symmetrical triazine looked promising: it noncompetitively inhibited folic acid[5] and competitively inhibited Crithidia factor (see Table VIII). Unfortunately this *s*-triazine had to be used at levels close to its solubility limit. A more convenient inhibitor is needed.

INHIBITION ANALYSIS AND BENZIMIDAZOLES

It has recently been noted[28] that benzimidazole inhibition of *E. gracilis* was not competitive; however, relationship of the inhibition to algal growth could be expressed graphically as a straight line when vitamin B_{12} concentration was 1×10^{-12} to 1×10^{-11} M and benzimidazole concentration was 10^8 times that of the vitamin B_{12}. One interpretation is that benzimidazole interferes with the ability of vitamin B_{12} to saturate some enzyme. It was not experimentally convenient to study competitive inhibition of purines by benzimidazole in *E. gracilis*, which has no exogenous purine requirement. We therefore shifted to *C. fasciculata* which does have this exogenous requirement and in which we found (Table IX), that inhibition between benzimidazole and adenine is noncompetitive.

Probably some reaction prior to closure of the purine ring or even the ring closure itself is competitively inhibited by benzimidazole. We think now that purine ring closure is pteridine-catalyzed. In the trypanosomids 4-amino, 5-imidazole carboxamidine participates in formation of the purine ring,[29] and this reaction is the most likely candidate for further investigation of the site of action of benzimidazole.

Additional targets of benzimidazole inhibi-

TABLE VIII

Competition Between a Triazine and Crithidia Factor in *Crithidia fasciculata*

Additions to pteridine assay medium	Crithidia factor[a] in μg %			
	0.01	0.03	0.10	0.30
None	0.68[b]	0.66	0.59	0.74
2,4-Dichloro-6-(o-bromo anilino) *S* triazine				
1.0 mg %	0.66	0.71	0.63	0.78
3.0	0.50	0.72	0.65	0.72
6.0	0	0.73	0.78	0.83
10.0	0	0.18	0.77	0.85
30.0	0	0.20	0.40	0.75

[a] 2-NH_2,4-OH,6-trihydroxypropyl pteridine was used as source of Crithidia factor.
[b] Growth as optical density units.

TABLE IX

Noncompetitive Inhibition of Adenine by Benzimidazole in *Crithidia fasciculata*

Additions to pteridine assay[a] medium	Adenine in mg %		
	0.1	0.3	0.6
None	0.57[b]	0.75	0.98
Benzimidazole			
0.03 mg %	0.50	0.72	0.86
0.6	0.50	0.74	0.86
1.0	0.62	0.73	0.74
3.0	0.26	0.32	0.34
10.0	0.20	0.20	0.22
30.0	0.04	0.07	0.08

[a] Complete except for purine source.
[b] Growth as optical density units.

TABLE X

Targets for Inhibition of *Crithidia fasciculata* by Benzimidazoles

Additions to pteridine assay medium	Supplements per 100 ml of medium			
	A		A plus	
	L-Glutamic acid	100.0 mg	Lecithin	1.0 mg
	Glycine	50.0 mg	Cholesterol	0.1 mg
	Purine mixture[a]	1.0 ml	Inositol	1.0 mg
	Pyrimidine mix.[a]	1.0 ml	Choline di-H citrate	1.0 mg
	Vitamin B_{12}	1.0 μg		
None	1.08[b]		1.28	
Benzimidazole				
6.0 mg %	0.98		1.25	
10.0	0.66		1.02	
30.0	0.25		0.37	
5,6-Dimethylbenzimidazole				
1.0 mg %	1.03		1.24	
3.0	0.89		1.08	
6.0	0.42		1.01	

[a] Described by Hutner *et al.*[16]
[b] Growth as optical density units.

TABLE XI

Targets for Inhibition of *Ochromonas malhamensis* by Benzimidazoles

	Additions per 100 ml of vit. B_{12} assay medium for *Ochromonas malhamensis*[a]		Benzimidazole in μg % None	1.0	3.0
No. 1	Vitamin B_{12}	10.0 μg			
	Purine mixture[b]	2.0 ml			
	Thymidine	2.0 mg	2.94[c]	0.22	0
	Na succinate	50.0 mg			
	Glycine	20.0 mg			
No. 2	Complete supplement No. 9[b]	5.0 ml	2.82	0.24	0
No. 3	No. 1 plus				
	Ergosterol	0.1 mg			
	Calciferol	0.1 mg	2.80	0	0
	Vitamin A acetate	1.0 mg			
No. 4	No. 1 plus lecithin	1.0 mg	2.93	1.62	0

[a] The assay medium for this organism usually is adjusted to pH 4.8–5.2. At such low pH, 0.1% benzimidazole is required for complete inhibition of *O. malhamensis*.[18] As shown in the table above, in media at higher pHs, lower concentrations of benzimidazole are inhibitory, suggesting better penetration of the drug. This medium, adjusted to pH 7.0–7.2, contained the following compounds (mg per 100 ml): $CaCO_3$, 3.0; $MgCO_3$, 30; KH_2PO_4, 20; metals 45A, 1.0 (composition given elsewhere[19]); HEDTA, 20; L-glutamic acid, 100; L-histidine·HCl, 100; L-arginine·HCl, 100; DL-methionine, 50; L-asparagine, 40; sucrose, 1000; succinimide·H_2O, 20; biotin, 0.0005; thiamine·HCl, 0.1; vitamin B_{12}, 0.0005; Tris, 100.

[b] Described by Hutner *et al.*

[c] Growth as optical density units.

tion in *C. fasciculata* were detected by adding more benzimidazole than known reversing agents could annul. Such inhibition was reversed by compounds not usually associated with vitamin B_{12} metabolism (Table X), and these reversing agents were not unique to benzimidazole inhibition in *C. fasciculata*. For example, lecithin reversed benzimidazole inhibition in *O. malhamensis* (Table XI).

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Hematopoietic Effects of Folic Acid Metabolites in the Megaloblastic Anemias

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MEGALOBLASTIC ANEMIA in man may result from a deficiency of either vitamin B_{12} or folic acid.[1,2] In many instances the presence of characteristic neurologic disease or of pronounced atrophy of the lingual papillae may make the diagnosis of vitamin B_{12} deficiency, pernicious anemia, virtually certain.[3] A history of dietary inadequacy in the absence of the neurologic or lingual manifestations of pernicious anemia, cheilosis, or a megaloblastic anemia with free hydrochloric acid in the gastric contents points to a deficiency of folic acid.

The diagnosis of vitamin B_{12} deficiency can be established by demonstrating on bioassay that there is little or none of the vitamin in the serum. Folic acid deficiency may be confirmed by measuring the excretion of formiminoglutamic acid in the urine after giving a loading dose of histidine orally.[4,5]

Occasionally patients with primary refractory anemia may have enough retardation in the maturation of the nuclei of the erythroid and/or myeloid cells of the bone marrow in comparison to that of the cytoplasm that vitamin B_{12} and folic acid deficiency must be excluded. In such patients there is no response to therapy, and in some frank myeloid leukemia eventually develops.

In 1955, we were stimulated by an influx of patients with various types of bone marrow failure to investigate in them the possible hematopoietic effects of nucleic acid precursors and derivatives. Although we have made little advance in the treatment of primary refractory anemia, and little progress in understanding its etiology in most instances some of the hematologic effects of exogenous metabolites in patients with megaloblastic anemia have been of fundamental interest. The manifestations of vitamin B_{12} deficiency in patients with pernicious anemia can be fairly regularly, but incompletely and only for some five to seven months, ameliorated by the oral administration of the pyrimidine precursor, orotic acid.[6] Since folic acid and vitamin B_{12} appear to have overlapping, parallel, or reciprocal functions, an investigation of the effects of the folic acid metabolites in the megaloblastic anemias was then begun. The present account is an interim report on a study that has not yet been completed.

Folic Acid Function

1 carbon transfer and incorporation.

Formyl-	Formimino-	Hydroxymethyl-
Histidine	Guanine,	Serine
Purine,	C_2	Methionine
C_2 and C_8		Thymidine

Fig. 1. Biochemical function of folic acid.

Since it was first discovered in spinach leaves, folic acid has been found most abundantly in liver, kidney, and fresh green vegetables. In

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the gastrointestinal tract and tissues it is reduced to tetrahydrofolic acid, in which form it serves as a coenzyme in many biosynthetic systems concerned with the transfer and incorporation of one-carbon fragments.[7] The latter include formyl-, formimino-, and hydroxymethyl groups (Fig. 1). There is evidence that a "one-carbon unit" pool exists in the body, and that there is some interconversion of the different groups. One-carbon moieties are required in the synthesis of several amino acids, the purine ring, and thymidine. Carbons 2 and 8 of purines are derived from a formyl group, as is the second carbon of histidine. The latter upon degradation may contribute to the formation of the purine ring.[8] In the synthesis of serine, methionine, and thymidine the methyl group is derived from a hydroxymethyl precursor which is transferred by an enzyme containing folic acid.

Individuals depleted of folic acid would be expected to have an impaired ability to synthesize some or all of these metabolites. If the now "essential" amino acid, purine, and thymidine requirements could be supplied adequately from exogenous sources, the nutritional defect might be overcome without replenishment of the missing vitamin or coenzyme. The relative importance of the folic acid metabolites in hematopoiesis, and possible interconversions of different compounds, might thus be studied in subjects with folic acid deficiency. Experiments were planned to test this hypothesis, utilizing patients with nutritional megaloblastic anemia.

The effect of exogenous pyrimidine and purine was studied in our first patient and that of amino acids in the second.

Case 1 (D 16383). E. M. J., a 30-year-old automobile salesman, attended the Duke Hospital Out-Patient Clinic occasionally after 1951 in reference to symptoms of emotional origin. Early in 1955 he began to lose weight from his average weight of 235 pounds to finally 180 pounds. In the fall of 1955 increasing weakness, pallor, dyspnea on exertion, palpitation, and edema of the ankles began to occur. On November 1, 1955, he was brought to the hospital a few hours after vision in his left eye became blurred. His family stated that he had used alcohol heavily for at least six to twelve months, and that his diet otherwise had consisted largely of "snacks," soft drinks, pastries, and candy.

On examination this young man was pale and anxious and showed evidence of weight loss. The blood pressure was 138/78 mm Hg. The sclerae were slightly jaundiced and flame-shaped hemorrhages were present in both optic fundi. The lingual papillae were about one-half normal height. The edge of his liver was palpable about 6 to 8 cm below the rib margins on the right; the spleen could not be palpated. There was pitting edema of both ankles. Neurologic examination showed no abnormalities.

Blood studies showed a hemoglobin concentration of 5.4 g per 100 ml; red blood cell count, 1,180,000 per cu mm; white cells 3600 per cu mm; hematocrit 15 per cent; and reticulocytes 2 per cent. In the stained films the erythrocytes appeared large and varied considerably in size and shape. Platelets were numerous and many of the neutrophils had multisegmented nuclei. Bone marrow aspirated from the sternum was extremely cellular and conspicuously megaloblastic.

Other laboratory studies showed a blood bilirubin level of 1.5 mg per 100 ml; total serum proteins, 5.3 g per 100 ml; and albumin, 3.5 g per 100 ml. Free hydrochloric acid was present in the gastric contents after histamine injection. The urine contained considerable urobilinogen but no bilirubin. On admission the bromsulfalein dye test of liver function, 45 minutes after the injection of 5 mg per kg, showed 32 per cent retention, but after three weeks' hospitalization this dropped to 10 per cent.

In the hospital the patient was offered a regular diet but he ate poorly. Vitamin supplements were withheld. The following experimental therapy was carried out: For a period of eight days he was given up to 6 g orotic acid per day orally (Fig. 2). There was a minimal rise in the reticulocyte percentage but no gain in hemoglobin, hematocrit, or red cell count.

He was then given thymidine intramuscularly 0.5 g daily for 14 days. The reticulocyte percentage rose to 14 per cent without clinical improvement or any other definite hematologic change. With the addition of inosine, 1.5 g daily administered intravenously, the reticulocyte percentage rose sharply to a crest of 22 per cent but then declined precipitously. There was a slight fall in hemoglobin concentration, hematocrit, and red blood count at this time. Methionine was then added in a dose of 6 g daily. His clinical and hematologic status became somewhat worse. On days 32 and 33 he was given folic acid, 5 mg each day, and thereafter 60 mg daily. There was a prompt and complete hematologic remission (see Fig. 2).

Case 2 (E 36927). V. McG., a 26-year-old Negro farmer's wife, was admitted to Duke Hospital on July 16, 1956. As a girl she was "chubby" and when she married at the age of 19 her weight was over 200 pounds. Her appetite continued to be robust and 5 years later she weighed over 300 pounds. She went through two pregnancies without complication. In May, 1955, when she became pregnant for the third time, she was advised to eat less fattening food, bread,

buttermilk, and sweets. Gestation progressed for several months without obvious complication. One week before delivery her physician saw her at home and advised her to restrict the salt in her diet. On February 15, 1956, he delivered an apparently normal infant weighing 8 lb 6 oz. She was taken home from the hospital three days later. At home she nursed the child for five months. It grew normally with breast milk being its only food. The patient, however, remained extremely weak and one to two months passed before she was well enough to be out of bed, up and around, for even a short time. In view of her continued weakness, she was advised to restrict her diet to fruit juice and buttermilk. Previously she had not drunk milk or eaten meat, except fish occasionally and one piece of chicken on Sundays.

Shortly before her admission to Duke Hospital she had a "spell" and was unable to move for a time. Her family physician was called. He discovered that she was very anemic, and referred her to the hospital immediately. At no time had she had a sore tongue, neurologic complaints, or abnormal gastrointestinal symptoms. Sixteen other people in the household were all well.

On admission to the hospital this young woman was pale, obese and lethargic. Blood pressure was 130/60 mm Hg. The temperature ranged from 38.2° to 39.4°C and the pulse from 95 to 125 per minute. The mucous membranes were extremely pale and the lingual papillae only about half as tall as normal. There was no cheilosis.

Blood studies showed a hemoglobin concentration of 2.0 g per 100 ml; red cell count, 1.6 million per cu mm; hematocrit, 8.2 per cent; and reticulocytes, 3.4 per cent. Bone marrow aspirated from the upper sternum was conspicuously megaloblastic.

As soon as she entered the hospital, she was given a 500-cc transfusion of packed red cells. Following this she was made comfortable and less febrile. She was then given a regular diet supplemented with 150 mg ascorbic acid daily but no folic acid or vitamin B_{12}. On the seventh hospital day when the hematologic status appeared to be stable, the oral administration of 0.5 g of DL-histidine, 0.5 g DL-serine, and 1.0 g methionine three times daily was begun and continued for one week. A prompt reticulocytosis occurred, with a crest of 35 per cent being reached on the sixth day (Fig. 3). The red cell count, hemoglobin, and hematocrit rose significantly. After the first week of therapy with amino acid, the DL-histidine alone was continued. She was given DL-serine 1.5 g. daily for a week subsequently without definite effect on blood status. When ferrous sulfate was added to the regimen, the hematologic status became normal (see Fig. 3).

Gastric analysis soon after admission to the hospital showed achlorhydria after injection of histamine. When repeated at a later date, free hydrochloric acid was present in the gastric contents.

Following a period of hematologic study she was seen infrequently. On a return visit 11 months after her first admission her physical and hematologic status

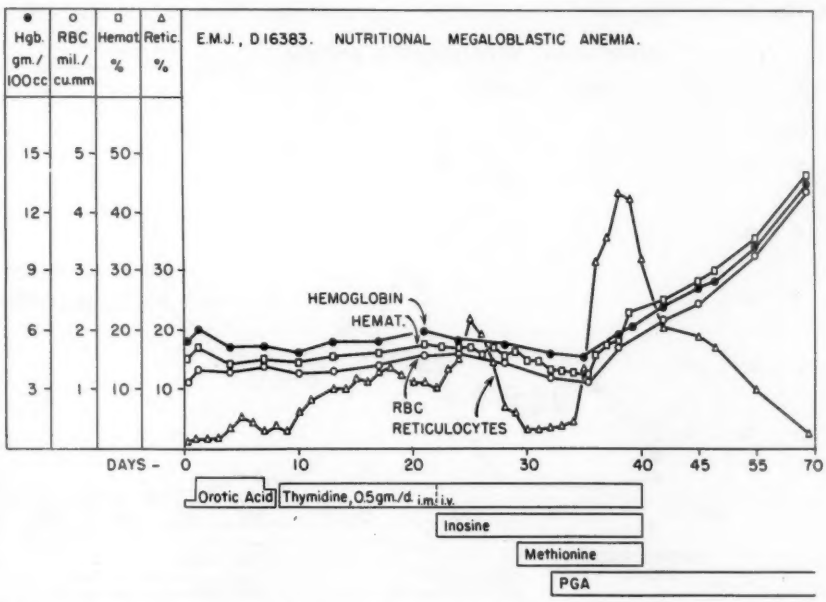

Fig. 2. Case 1. Hematologic response of patient with nutritional megaloblastic anemia to administration of orotic acid, thymidine, inosine, methionine, and folic acid (PGA).

appeared to be satisfactory. She had returned meanwhile to her earlier dietary habits.

DISCUSSION

Patients with megaloblastic anemia may have a significant hematologic response to the administration of amino acids, purine, or pyrimidine compounds normally synthesized endogenously by folic acid-containing enzymes. The total potential effects of each metabolite, given singly or in combination to patients with either folic acid or vitamin B_{12} deficiency, remains to be ascertained.

Three patients with nutritional megaloblastic anemia, in addition to the two cited in detail herein, have been available for study. Histidine, serine,[9] and formiminoglycine when given alone have produced suboptimal hematologic responses. No combination of metabolites used so far, in doses that have been practical, has reproduced the complete therapeutic effect of folic acid.

Five patients with pernicious anemia have been studied since the group previously reported.[6] The administration of serine, methionine, and formiminoglycine have failed to produce significant hematologic responses, and thymidine has shown little effect.[10] DL-histidine, administered in doses of 3 to 4.5 g daily, has usually produced some reticulocytosis, and one patient had an excellent response which was maintained over a period of at least four months on a dose of 10 to 20 g daily.

These studies when complete may yield information regarding the function of vitamin B_{12} in man, which at this time is obscure.[11] Folic acid antagonists have been of considerable importance in the therapy of leukemia. It is conceivable that the antileukemic effect does not depend upon blocking all folic acid functions. The antileukemic mechanism could be investigated in more detail and, if better understood, irrelevant toxic reactions might be counteracted by the simultaneous administration of one or more of the folic acid metabolites. The antifolic compounds could then be used with greater selectivity and efficiency. Finally, the possibility that the bone marrow proliferation defect in primary refractory anemia may be corrected by the administration of exogenous metabolites awaits further investigation.

SUMMARY

Folic acid functions as a coenzyme in the biosynthesis of three amino acids, serine, histi-

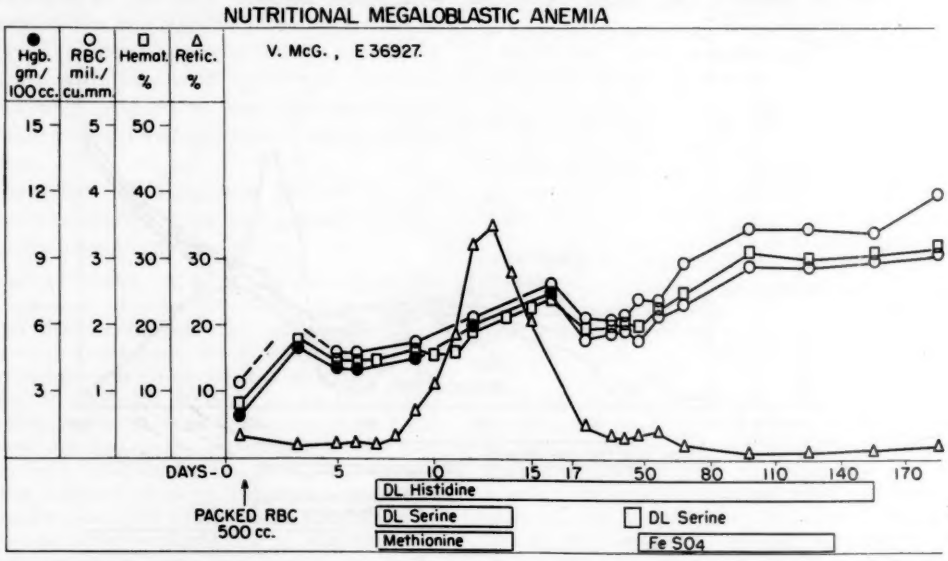

Fig. 3. Case 2. Hematologic response of patient with nutritional megaloblastic anemia to amino acids dependent upon folic acid in biosynthesis.

dine, and methionine, the purine ring and thymine. The major clinical manifestation of folic acid deficiency is megaloblastic anemia, presumably due to a lack of these "essential" metabolites.

The hematopoietic effect of folic acid metabolites when administered singly and in combination to patients with megaloblastic anemia has been studied. Significant but usually suboptimal responses have been obtained so far with serine, histidine, inosine, and thymidine.

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Nutrition and Disease: Folic Acid Deficiency in the Mouse

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IT HAS BEEN generally accepted that the growing mouse fed a purified diet low in folic acid would not become deficient in folic acid unless a vitamin antagonist, low casein diet, stress agent, or intestinal germicidal agent were given.

For example, Franklin, Stokstad, and Jukes,[1] in 1947, fed mice diets low in folic acid without obtaining deficiency signs unless a folic acid antagonist was given. In their experiments even the addition of sulfasuxidine to the diet did not result in a deficiency. In 1948 a folic acid deficiency was produced in mice by Weir, Heinle, and Welch[2] by the addition of a crude folic acid antagonist to the diet. These authors also observed changes in the blood components. Woolley[3,4] reported that mice grew normally on a purified diet containing the then known B vitamins (not including folic acid) and inositol.

In 1944, Cerecedo and Vinson[5] reported that they were able to rear three strains of mice through several generations on highly purified diets. Their diets contained casein, salts, fiber, fat, sucrose, and all vitamins except folic acid. Growth without folic acid was actually superior to that of control mice kept on stock diets. However, reproduction and lactation were inferior in the mice fed the purified diet unless a concentrate of folic acid was supplied. Later, in 1947, Cerecedo and Mirone[6] demonstrated that the addition of crystalline folic acid to the diet had a similar effect.

Fatterpaker and co-workers[7] produced a folic acid deficiency in mice on diets low in casein and containing 0.3 per cent of iodinated casein. In 1957 Schneider, Lee, and Olitsky,[8] reported that mice grew normally when fed diets low in folic acid (and biotin and vitamin B_{12}), although they did find that the addition of these three vitamins, alone or together, restored the susceptibility of the mouse to acute disseminated encephalomyelitis. A large number of studies have been reported in the cancer journals on various effects of folic acid antagonists in the mouse. No attempt is made here to review such studies.

Saucier and Demers[9] reported that mice fed diets low in casein and choline grew poorly and had anemia and hypercholesterolemia unless folic acid or vitamin B_{12} were given.

Nielsen and Black[10] in 1944 published what appears to be the only previous report of the production of a folic acid deficiency in mice fed normal purified diets (with 20 per cent of casein). Mice 7 to 9 g in weight were used in experiments lasting eight weeks. Differences of 6 to 7 g were noted in this time. Unfortunately, pure folic acid was not available to prove that the results could be attributed to folic acid (a crude liver preparation was used).

This paper presents full details of our nutritional procedures and results, and reviews our recent studies[11–13] in which we have found means of routinely producing an "uncomplicated" folic acid deficiency in the mouse without the use of an antagonist or sulfa drug. In addition, these studies have provided a new tool for the study of certain relationships between nutrition and disease as will be described.

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TABLE I

Composition of Control Diets with Folic Acid

Constituent	Diet M4 g/kg	Diet C2 g/kg	Diet M9 g/kg
Glucose (cerelose)	538	615	610
Casein (vitamin-free)	200	200	200
Gelatin	—	80	80
DL-methionine	—	3	8
Corn oil	20	40	40
Hydrogenated vegetable oil (Crisco)	180	—	—
Salts A[14]	60	60	60
Water-soluble vitamins[a]	2	2	2
Fat-soluble vitamins[b]	mg/kg	mg/kg	mg/kg
Vitamin A acetate	6	3	3
Vitamin D_3	0.04	0.02	0.02
α-Tocopherol acetate	20	10	10
2-Methyl-1,4-naphthoquinone	2	1	1
TOTAL	1000 g	1000 g	1000 g

[a] The water-soluble vitamins were added in the form of a premix of glucose as follows (mg/kg of diet): thiamine HCl 8, riboflavin 8, calcium pantothenate 20, nicotinic acid 100, pyridoxine HCl 8, D-biotin 0.3, vitamin B_{12} 0.02, and pteroylglutamic acid (folic acid) 3.0 when indicated. Crystalline choline chloride was also added at a level of 2 g/kg of diet. The glucose in the premix was included in the total amount of glucose shown in the table.

[b] The fat-soluble vitamins were added from a stock solution in corn oil (in such amounts that 1 per cent of corn oil supplied the amounts indicated). Vitamins A, D, and K were kept in one stock solution and vitamin E in another. The stock solutions were kept refrigerated.

EXPERIMENTAL PROCEDURES

Unless otherwise indicated, C57 black male mice weighing 8 to 10 g were used in all the dietary studies. They were obtained from the National Institutes of Health colony. The mice were kept in stainless steel "hanging" cages with wire screen bottoms. Each group consisted of six mice housed in one cage. The mice were fed three times a week or more often if needed. The feed cups were constructed in such a manner that feed wastage and feed contamination were kept at a minimum. Experiments were conducted for a six-week period unless otherwise indicated. Weighings were made once each week.

The composition of the diets used in these experiments is given in Table I. No folic acid antagonists or sulfa drugs were used in these diets unless otherwise indicated. All diets were kept refrigerated until used, thus making it possible for sufficient diet to be mixed at one time to last through an entire six-week experiment.

The method of administration of the lymphocytic choriomeningitis virus has been previously described.[12]

RESULTS AND DISCUSSION

In our early studies we found that folic acid deficiency was not produced in mice by removing folic acid from our standard laboratory mouse diet, M4. This is comparable to the experience of others. However, in our studies with chicks we had been routinely very successful in obtaining a folic acid deficiency by diet alone (diet C2). For this reason we fed mice the chick diet which was low in folic acid (diet C2 minus folic acid); rather unexpectedly this resulted in a retarded growth rate in a total of three experiments unless folic acid was present. The major difference in these two diets (see Table I) were in the levels of fat and fat-soluble vitamins and the presence of added gelatin and methionine in the chick diet (diet C2). It was soon determined that the presence of the gelatin and the methionine accounted for the folic acid deficiency in the mouse produced by feeding the chick diet, C2. A level of 0.8 per cent of methionine appeared to be best for the production of a folic acid deficiency in the mouse, and this level was used in all succeeding experiments.

Further preliminary studies to find the amino acid(s) in gelatin responsible for producing the deficiency showed that by merely adding 1.7 per cent of glycine (the amount supplied by 8 per cent of gelatin) to the mouse diet (M4) a good folic acid deficiency could be produced in the presence of methionine. So as not to cause too much of an amino acid imbalance, gelatin was used in all of the following experiments instead of pure glycine. The level of fat and fat-soluble vitamins in the diet was not a significant factor in the production of a folic acid deficiency. Therefore, in formulating the mouse diet (M9) which was used in all succeeding studies, these nutrients were maintained at the same level as in diet C2. In fact, the mouse diet M9 is the same as the chick diet, C2, except for the change in the level of methionine.

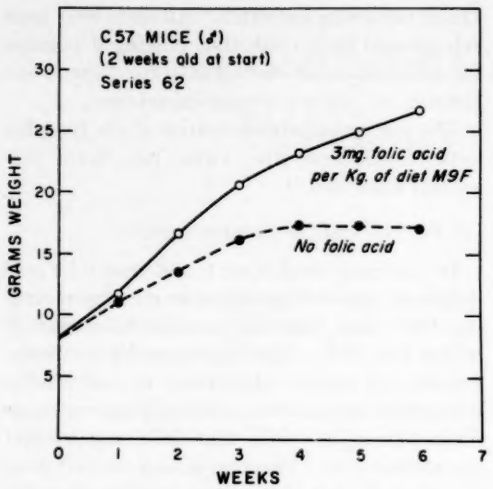

Fig. 1. Growth curves of folic acid deficient mice on diet M9 as compared with growth of control mice. There was no mortality in this series.

When C57 male mice were fed diet M9 minus pteroylglutamic acid they grew at a rate about equal to that of control mice fed pteroylglutamic acid for one or two weeks and then, as in a typical experiment shown in Figure 1, grew at a slower rate. At about four weeks of age a growth plateau was reached in nearly all experiments. In 17 of 20 different experiments of six C57 male mice per group, deficient mice weighed an average of 15 g at the end of six weeks, whereas control mice receiving 3 mg of pteroylglutamic acid per kg grew normally, had no mortality, and weighed an average of 25 g. (Female C57 mice behaved similarly in two six-week experiments.) Mortality within individual deficient groups varied—usually all mice survived but in a few instances most mice in a group of six were dead by six weeks. Contrary to these results, in 3 of 20 experiments, deficient mice weighed as much as the control mice at six weeks of age. The significance of this is discussed later.

We have kept folic acid deficient mice on the deficient diet for longer periods of time than six weeks with varying results. Sometimes there was a high death loss in several weeks and in some instances the entire group of six mice lived for many months. Such results indicated to us early in the experiments that the deficient animals live in quite a delicate balance with respect to synthesis of folic acid by intestinal microorganisms or by some other mechanism. It was determined rather early in our studies that it was important to clean the mouse cages and feeding equipment whenever they became contaminated with fecal matter; coprophagy appeared to be a factor in causing the variability between experiments. Variability was kept at a minimum when this was done and greater differences were obtained with and without folic acid.

TABLE II

Effect of Age of Mouse on Folic-Acid Deficiency

Age of mouse 1 *wk*	Weight at start *g*	Diet	Weight after 6 wk on experiment *g*
2	7.4	M9 minus folic acid	12.6 ± 1.0
2	7.4	M9 plus 3 mg folic acid/kg	22.5 ± 0.3
3	12.8	M9 minus folic acid	22.3 ± 0.3
3	12.8	M9 plus 3 mg folic acid/kg	22.8 ± 0.6

Factors in Production of Folic Acid Deficiency

Effect of Age of Mouse: It was found early in these studies that the age and size of the mouse at the time of starting the experiment were important in determining the results obtained. Table II gives the results of two experiments on six mice each. Although a deficiency of folic acid has been produced in our laboratory in older mice over 12 g in weight, the deficiency is more consistently obtained with smaller mice. We now routinely use mice 8 to 10 g in weight.

Effect of Strain of Mouse (Genetics): To find whether or not the genetics of the mouse were responsible for our unexpected results, several strains of mice produced in the National Institutes of Health colony were fed the folic acid deficient diet (M9 minus folic acid) and compared with control mice. The results are shown in Table III. It is clear that all strains of mice tested became folic acid deficient as evidenced by reduced growth rates and increased mortality rates. It is assumed, therefore, that differences in strain of mice used in the various laboratories are not responsible for the differences in experimental results.

TABLE III

Effect of Genetics on Folic Acid Deficiency in the Mouse[a]

Strain	Supplement to diet M9[b]	Weight after 6 wk ± S.E. g	Mortality/ no. mice
C57B1	None	17.1 ± 1.0	3/12
	Folic acid	24.5 ± 0.5	0/12
NIH general-purpose (Swiss)	None	20.6 ± 1.2	4/12
	Folic acid	22.6 ± 0.8	0/12
CFW	None	20.2 ± 1.8	5/12
	Folic acid	28.3 ± 0.6	1/12
DBA	None	12.0 ± 1.5	10/12
	Folic acid	23.9 ± 0.4	1/12
A/LN	None	14.9 ± 1.0	2/12
	Folic acid	23.0 ± 0.5	0/12
CDBA	None	20.0 ± 1.0	0/12
	Folic acid	26.0 ± 0.4	0/12

[a] All groups averaged approximately 8 g at start of experiment and 12 male mice were used in all instances (average of two experiments).

[b] Control mice were all fed 3 mg of pteroylglutamic acid (folic acid) per kg of diet.

Effect of Various Substances on Growth of Deficient Mice: Various compounds have been tested for their effect on growth of deficient mice. A considerable number of substances have had no effect on growth when added to the diet of deficient mice in various experiments (details not given to conserve space). These include: Aureomycin® (50 mg/kg of diet), cellulose (15 per cent), Crisco (up to 20 per cent), ascorbic acid (0.5 per cent), cystine (1 per cent, proline (1 per cent), isoleucine (1 per cent), tryptophan (0.2 per cent), phenylalanine (0.5 per cent), histidine (0.5 per cent), various mixtures of these amino acids, and 0.1 per cent each of several purines and pyrimidines (adenine, guanine, thymine, orotic acid, uridylic acid, and cytidine).

Several substances have increased the severity of the deficiency, including additional glycine, gelatin (up to 15 per cent), and sulfasuxidine (0.5 per cent), as would be expected.

On the other hand, several substances have a marked sparing effect on folic acid under these conditions. These include procaine penicillin G, which at a level of 50 mg/kg of diet completely replaced the need for folic acid in three experiments.* Also, para-aminobenzoic acid, which is a constituent of pteroylglutamic and is known to spare folic acid in other animals, had a marked sparing effect on folic acid (see Table IV for results of a typical experiment). The exact folic acid requirement has not been determined under these conditions but it appears to be less than 0.5 mg/kg of diet. Para-aminobenzoic acid was not as active as folic acid, but the exact relationship needs further study. Calcium leucovorin (Lederle) was fully as active as pteroylglutamic acid when compared at low levels in the diet. This was rather unexpected, since in the chick calcium leucovorin is not as active as folic acid when present in the diet.[14]

TABLE IV

Comparison of Activity of Folic Acid and Para-aminobenzoic Acid (PABA) in Male C57 Mice

A typical experiment

Supplement to diet M9 minus folic acid	No. mice	Mortality at 6 wk	Weight at 6 wk ± S. E. g
None	6	0	16.8 ± 0.7
1 mg PABA/kg	6	0	22.9 ± 0.9
5 mg PABA/kg	6	0	24.3 ± 0.7
50 mg PABA/kg	6	0	24.8 ± 0.4
0.5 mg PGA/kg	6	0	24.8 ± 0.7
1.0 mg PGA/kg	6	0	25.0 ± 0.5
3 mg PGA/kg	6	0	24.8 ± 0.4

Observations Related to Folic Acid Deficiency

Excretion of Formiminoglutamic Acid: In a trial with pooled urine from six mice on a deficient diet a total of 11.9 micromols of formiminoglutamic acid was excreted in a 24-hour period, whereas none was excreted in control animals fed 3 mg of pteroylglutamic acid per kg.† This is similar to results which have been obtained with rats fed diets low in folic acid.[15]

Other Observations: There was no change in the black hair color in the deficient mice. Depigmentation might have been expected from studies with folic acid in other animals. Alopecia was observed only rarely and was not apparently correlated with changes in the diet.

* Studies made in cooperation with Dr. Victor H. Haas. It is possible that the procaine portion of the molecule is responsible for this effect because of its relationship to PABA.

† I wish to thank Dr. Milton Silverman and Miss Rita Gardiner for making these assays.

Mice on the deficient diet for extended periods of time showed gross evidence of anemia. Preliminary blood studies on four deficient mice showed a significant drop in hemoglobin as compared with control mice. Complete blood studies were made by Drs. Martin Leibling and Emil Frei and will be reported elsewhere.

Effect of Folic Acid Deficiency

Relationship to Virus Infection: Folic acid deficient mice obtained in these studies have been used in experiments conducted in cooperation with Drs. V. H. Haas and S. E. Stewart. The results of these experiments have been published[12] and are only briefly described here.

It has been shown that the injection of amethopterin, a folic acid antagonist, prevented death of mice given an intercerebral injection of the virus of *lymphocytic choriomeningitis* (LCM) in doses that killed all untreated control mice.[16] The spared mice had prolonged viremia and developed immunity. It was of interest, therefore, to see whether similar results could be obtained in mice made folic acid deficient by dietary means without the use of drugs. It was found that death from LCM injections did not occur in mice fed the low folic acid diet M9 for a six-week period. This is similar to the results with amethopterin. Control mice, fed 3 mg pteroylglutamic acid, were all killed by similar injections. Details of three typical experiments are shown in Table V. Similar results have been obtained in other trials. It is obvious, therefore, that the effect of amethopterin is truly due to its folic acid inhibiting effect and not to any other nonspecific effect it might have.

It is of interest that in mice in which death was prevented by folic acid deficiency or by amethopterin an immunity developed after several weeks.[11,12] These mice could be placed later on a normal stock diet with folic acid and would live indefinitely. Active virus, capable of killing other mice, was obtained from the brains of the spared mice as late as the twenty-ninth day. Thus, a high level of virus production occurs in the spared mice. Apparently, in the absence of excess folic acid in the brain, an immunity develops before the virus has a chance to kill the animal.[12] The exact explanation is not known.

TABLE V

Prevention of Death from LCM Injections in Mice Fed Folic Acid Deficient Diets[12]

C57 male mice, 6 weeks on deficient diets

Diet	Experiment	Average weight g	No. mice dead by 14 days/ no. injected	Average day of death
M9 minus folic acid	1	12	0/5	—
	2	22	0/6	—
	3[a]	19	0/6	—
M9 + 3 mg folic acid/kg	2	23	6/6	8.3
	3[a]	21	6/6	8.3

[a] In experiment 3, diet M4 (see Table I) plus 8 per cent of gelatin was used.

It is an interesting observation that the effect of a folic acid deficiency in preventing death was noted in several experiments, with mice which had shown no weight differences compared to control mice. This is a good argument for not using weight differences as an essential criterion of a nutrient deficiency in experimental nutrition studies. In this instance, for example, the effect of a folic acid deficiency in preventing death from LCM virus provides a tool by which a folic acid deficiency can be diagnosed in the mouse when other means, including weight changes, are not available. We have used this tool successfully in other studies (not described here) with folic acid deficient mice.

These results in experiments 2 and 3 (see Table V) also provide good evidence that possible inanition in the deficient mouse was not a contributing factor in the results obtained. The deficient mice in these experiments were eating well throughout the study.

Relationship to Transplantable Lymphocytic Neoplasms: Because of the interesting results with LCM virus it was obvious that similar studies should be made with certain forms of mouse leukemia (lymphocytic neoplasms) known to be sensitive to amethopterin. These studies have been made with Dr. M. Potter of the National Cancer Institute and will be published in detail elsewhere.

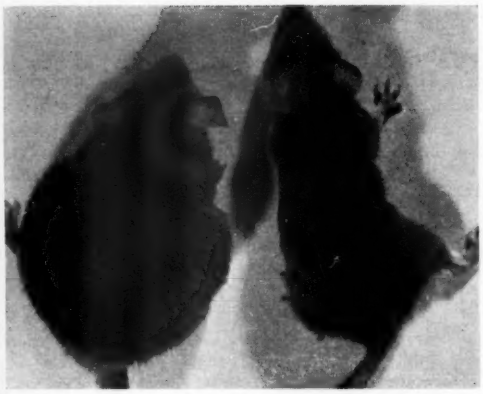

Fig. 2. Typical mice from the studies on lymphocytic neoplasms in folic acid deficiency. Both mice were injected with ascitic tumor cells 14 days before the photograph was taken. The mouse on the left received folic acid and died on day 15. The mouse on the right received the deficient diet and lived 8 days longer.

In brief, ascitic tumor cells of certain transplantable lymphocytic neoplasms (P288, P288AMRII, P388), in varying inocula size, were injected intraperitoneally into groups of hybrid (BALB/c × DBA/2) F1 mice.[17] In mice fed the deficient (M9) rations the onset of ascites and death were significantly delayed in contrast to the control mice, as demonstrated by Figure 2. One unexpected finding was that an amethopterin-resistant tumor responded in the same manner as an amethopterin-sensitive tumor. These details are quite complex and many variables affect the results. Further studies are in progress.

By means of studies such as these it should be possible to use intact animals fed normal diets without drugs to study nutritional factors which affect virus multiplication or growth of neoplasms. It is hoped that additional work will provide solutions to the problems raised here.

SUMMARY

An uncomplicated deficiency of folic acid has been produced by dietary means in young mice of various strains in three to six weeks without the aid of a folic acid antagonist or a sulfa drug. This has been done by keeping mice in cages with screen bottoms (to prevent coprophagy) and feeding them a synthetic diet low in folic acid containing 20 per cent casein, 8 per cent gelatin, and 0.8 per cent methionine, in addition to the other usual ingredients. The deficiency may be overcome by feeding 0.5 mg or more pteroylglutamic acid per kg of diet or by feeding larger amounts of para-aminobenzoic acid or procaine penicillin G.

The folic acid deficient mice have been used to study the relationship of nutrition of infection with the virus of lymphocytic choriomeningitis and to certain transplantable lymphocytic neoplasms. With both types of infection the time of death was significantly delayed in folic acid deficient mice. These studies provide a new tool which may be used to study the relationships between folic acid and disease in normal intact mice not receiving drugs.

ACKNOWLEDGMENTS

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Urinary Excretion of Formiminoglutamic Acid

Application in Diagnosis of Clinical Folic Acid Deficiency

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ALTHOUGH folic acid, an essential nutrient, was isolated more than 15 years ago,[1,2] methods for the evaluation of folic acid deficiency left much to be desired. Because deficiencies of folic acid or vitamin B_{12}, result in megaloblastic anemias, morphologically identical, identification of folic acid deficiency, in the absence of central nervous system disease, was possible only by therapeutic trial.[1-3] Failure of therapy with vitamin B_{12}, but a subsequent dramatic response to the administration of relatively minute doses of folic acid, indicated folic acid deficiency.[3] Until recently there have been no rapid and reliable methods for the evaluation of folic acid deficiency. The urinary excretion of formiminoglutamic acid (FIGLU), however, under the conditions described herein, can apparently be employed as a sensitive, reliable and rapid test for biochemical folic acid deficiency states in man.

Available data regarding folic acid blood levels as a criterion of folic acid nutrition are confusing. Different laboratories report values from below measurable amounts to 15 mμg/ml in normal subjects.[4-8] The use of folic acid serum, plasma, or whole blood levels as an index of folic acid deficiency has also been inadequate, because the microbiologic assays available are not sufficiently specific. Purines, pyrimidines, folic acid analogues such as folinic acid, pteroic acid, and still unidentified factors[8] normally present in blood, may replace the folic acid growth requirement of the test microorganisms. Usdin, Phillips, and Toennies[8] were unable to detect folic acid, folinic acid, or any related known analogue in whole blood. They found that the material in whole blood which shows folic acid activity by microbiologic assay contains at least six as yet unidentified components. Chanarin has recently re-emphasized these problems.[9] Chanarin, Anderson, and Mollin,[10] found no detectable folic acid in the serum of 55 *normal* subjects tested with *Streptococcus faecalis*, using a modification of the method of Teply and Elvehjem[11] as reported by Jukes.[12] The recently proposed clinical usefulness of the L. casei folic acid assay requires further evaluation.[3,33] The leukocyte folic acid concentration in patients with megaloblastic anemias, presumably due to folic acid deficiency, has not been of value as an index of clinical folic acid deficiency because the differences from normal were not sufficiently discriminatory.[34]

Methods for the evaluation of gastrointestinal absorption of folic acid include determination of its urinary excretion following the administration of a "load" dose of 3 to 5 mg of folic acid either before or after "saturation" of the patient with 3 to 5 mg injected subcutaneously for several days prior to the test. This test was found by Girdwood[14] to differentiate patients with malabsorption syndromes from normal persons. Others,[10] however, have found

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sufficient overlap between the results of the urinary excretion test in control subjects as compared with the results in patients with malabsorption syndromes to render interpretation of results difficult. Study of the gastrointestinal absorption of folic acid by determining the serum concentration of folic acid, after saturation with folic acid, and then following a 3 mg oral dose, has been found by Chanarin and associates[10] to be more definitive than the Girdwood urinary excretion test for detection of malabsorption in idiopathic steatorrhea. It was not useful in other types of malabsorption syndromes. These gastrointestinal absorption tests, however, are not useful for evaluation of folic acid nutrition, as patients with normal results may have evidence of megaloblastic anemia which requires treatment with folic acid.[10]

A clue which led to the development of a direct biochemical test for folic acid deficiency was the observation by Bakerman, Silverman, and Daft[15] that rats made deficient in folic acid excreted large amounts of a glutamic acid precursor in their urine. Further studies[16,17] revealed the glutamic acid precursor to be formiminoglutamic acid (FIGLU), and the extent of its urinary excretion paralleled the degree of folic acid deficiency of the experimental animals. Subsequent investigations by this group and others[17–21] demonstrated that in animal and bacterial systems tetrahydrofolic acid was a necessary coenzyme for the conversion of FIGLU to glutamic acid. Tetrahydrofolic acid arises from dietary sources of folic acid. It is the physiologically active form of folic acid which removes the formimino group from FIGLU, converting the latter to glutamic acid and eventually, by the steps illustrated in Figure 1, making the carbon unit of the formimino group available to the metabolic pool for synthesis of amino acids and purines, after liberation of the nitrogen as ammonia.

FIGLU was first detected by microbiologic

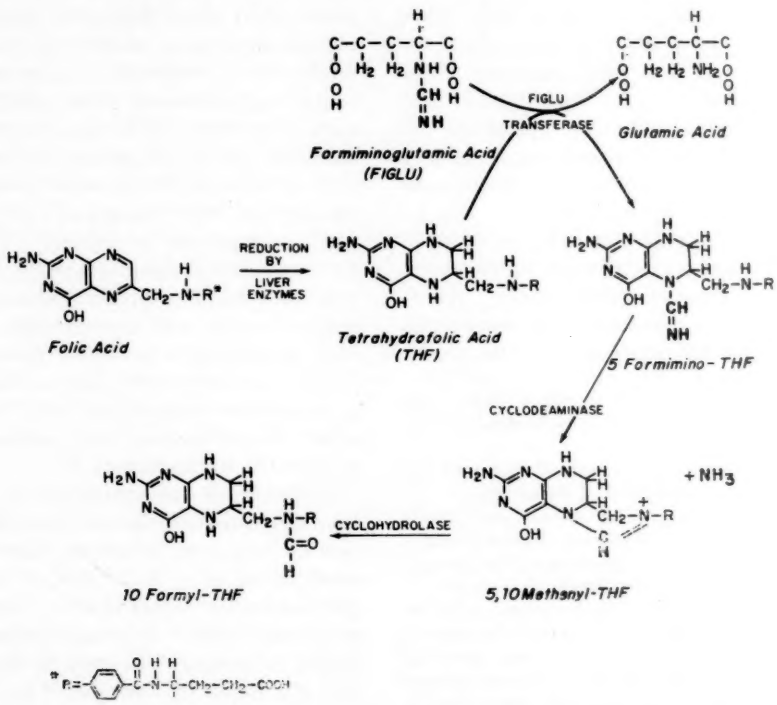

Fig. 1. Degradation of FIGLU to glutamic acid and role of folic acid coenzymes in one carbon unit transfer.

assay in the urine of human subjects treated with folic acid antagonists and in patients with the macrocytic anemia of pregnancy by Broquist[22] and Luhby.[23] Further observations in our laboratory[24,25] confirmed these findings and extended them to a variety of other clinical folic acid deficiency states. The compound detected by microbiologic assay in the urine of a patient receiving amethopterin was subsequently isolated and shown to be identical with synthetic FIGLU.[26]

In our hands the determination of urinary FIGLU has proved to be a sensitive and specific determinant of clinical folic acid deficiency. In this paper the methods for the assay of FIGLU and their application to the diagnosis and assessment of folic acid deficiency states in man are described.

ASSAY METHODS

Microbiologic Assay

When these studies were initiated, the microbiologic assay was the only quantitative method available. This assay utilizes the semimicro method of Henderson and Snell[27] for glutamic acid. The bacterium *Lactobacillus arabinosus* is grown in a synthetic nutrient broth which contains all the known growth factors for this organism with the exception of glutamic acid. Under these conditions, there is a quantitative relationship between the amount of "glutamate," i.e., glutamic acid or glutamine, added to the assay medium and the growth of the microorganism. FIGLU, as such, does not support its growth. However, when a solution of this compound is heated, it is degraded to glutamic acid, which permits growth. This heating process simultaneously converts the glutamine normally present in urine to pyrrolidone carboxylic acid, which is incapable of supporting growth. These relationships are illustrated in Table I.

TABLE I

Growth Response of *L. Arabinosus* (in a Glutamic Acid-free Medium) to FIGLU, Glutamic Acid, and Glutamine

	FIGLU	Glutamic acid	Glutamine
Unheated	No growth	Growth	Growth
Heated	Growth	Growth	No growth

The increase in growth of the culture is proportional to the turbidity which is read in a colorimeter against appropriate blanks. Results are expressed as the ratio of the concentration of "glutamate" in a heated and unheated aliquot of the urine being assayed, H/U ratios of 0.9 or greater usually indicate significantly increased quantities of FIGLU are present.

The method has a sensitivity of about 10 μg of "glutamate activity" per ml. It is reasonably specific. Reliable and reproducible results are obtainable. Its great advantage is that the reagents necessary are readily available and the technic does not require elaborate or expensive equipment.

The disadvantage of the method is that the assay requires 72 to 96 hours for completion. It also necessitates conversion of the FIGLU to glutamic acid by autoclaving, a time-consuming preparatory procedure.

In addition, *if the glutamine content of the urine is low, misleadingly high* H/U *glutamate activity ratios are obtained when the actual quantity of* FIGLU *is not greatly increased above normal.* We have also found that the dark color of many urine specimens often results in falsely high values, despite the use of an appropriate urine blank. To overcome the latter difficulty, we have adopted charcoal decolorization of all urine samples prior to assay. This treatment was found not to affect the amino acid content of the urine and markedly improved the reliability of the assay. Preassay charcoal treatment also removes amethopterin or aminopterin from urine[26] of patients receiving folic acid-antagonist therapy which would interfere with the growth of the assay organism.

Urine of pH 5 or higher containing FIGLU may be kept for short periods at −10° to −25°C without loss of activity. However, if many samples are to be assayed, this requires considerable deep-freeze storage space. Such urine specimens cannot be rechecked after several weeks to months, and it may not always be convenient to perform an assay soon after collection. We have found that addition of 1 ml concentrated hydrochloric acid per 100 ml of expected urine added to the collection con-

tainer to maintain a pH of 2 or less preserves the stability of FIGLU in such urine samples for at least one year at room temperature.

Paper Chromatography Assay

FIGLU moves faster in a descending solvent front paper chromatogram than either glutamic acid or glutamine and has R_f values sufficiently different from other amino acids in a number of solvent systems to make paper chromatography a suitable assay procedure.

The technic is simple and rapid. Only 18 hours is required for completion. We have found that a solvent system consisting of 15 per cent formic acid, 70 per cent *t*-butanol, and 15 per cent water[28,29] is suitable and preferable to the more corrosive or malodorous mixtures containing phenol[22] or collidine.[26]

Because FIGLU contains no free amino group, it is not colored by ninhydrin. Exposure of the paper strips containing FIGLU to ammonia vapor hydrolyzes off the formimino group, frees the amino group, and permits ninhydrin color development. This feature adds considerably to the specificity of the assay. Quantitation of the spots is possible by use of a densitometer.

The major disadvantage we have experienced in the use of this assay is that its sensitivity, in the range of 20 to 50 μg FIGLU per ml, is poor compared to the other available methods. In addition, urine specimens often contain compounds with R_f values so close to FIGLU that differentiation becomes difficult and specificity is compromised. The latter difficulties could probably be resolved by employing two-dimensional paper chromatography. However, introduction of the enzyme assay of Tabor and Wyngarden[30] precluded further development of the chromatographic assay at this time. Paper chromatography is useful, nevertheless, for qualitative purposes and as an alternative identification method.

Enzyme Assay

In this procedure, a modification of that introduced by Tabor and Wyngarden,[30] urine samples containing FIGLU are incubated with tetrahydrofolic acid and a semipurified liver concentrate in an appropriate buffer. The reaction mixture yields 10-formyltetrahydrofolic acid which upon acidification is converted to 5,10-methenyltetrahydrofolic acid. The latter compound can be quantitated in a Beckman DU spectrophotometer at its maximum absorption wavelength of 350 mμ in the ultraviolet.

This enzymatic assay for FIGLU is the most sensitive and specific technic currently available. It is a direct method, requires little preparation of samples and yields results readily in 5 to 6 hours. As little as 0.1 μg FIGLU per ml can be detected. These are outstanding advantages, particularly for a laboratory in which many samples are assayed daily, and results may govern the experimental procedure of the following day.

The chief disadvantages of this technic are the expensive apparatus required and the need for laborious preparation of reagents not commercially available.

Enzyme-Microbiological Assay

A method for the determination of FIGLU utilizing a combination of enzymatic digestion and microbiologic assay of urine was developed by Silverman *et al.*[31] This is an indirect method which requires (1) the enzymatic reduction of folic acid to tetrahydrofolic acid (THF); (2) the enzymatic formylation of THF by the FIGLU in test urine; (3) the nonenzymatic conversion of the latter product, N^{10}-formyl THF to N^5-formyl THF (citrovorum factor); and (4) the assay of the N^5-formyl THF microbiologically.

This method is time-consuming, necessitating elaborate preparation of samples and requiring 36 to 48 hours for completion of the assay. This assay is more sensitive than the *L. arabinosus* microbiologic assay, but may lack specificity. Urocanic acid and perhaps other intermediates, which do not affect the all-enzyme assay, are equally as active as FIGLU and may give false values in this method. Urine samples are therefore assayed before and after autoclaving. Since the latter procedure destroys FIGLU, the difference between the heated and the unheated aliquot represents FIGLU.

CLINICAL MATERIAL AND METHODS

Excretion of FIGLU in the urine was studied in patients with a variety of megaloblastic macrocytic anemias and following folic acid-antagonist therapy. Control subjects included patients with other types of anemia as well as those with hematologic normality and no apparent disease. Urine specimens were collected at the bedside in bottles containing sufficient concentrated hydrochloric acid to maintain the pH at 2 or less, as already described. Urine specimens before and after treatment were collected on a 24-hour basis whenever possible. Assay for FIGLU was performed by a modification of the enzyme method of Tabor and Wyngarden[30] or the *L. arabinosus* microbiologic assay for glutamate[26] described previously. FIGLU results were calculated in micrograms per milliliter and milligrams per 24-hour urine output. Results of the microbiologic assay were expressed as the ratio of the heated to the unheated (H/U) urine assay. Metabolic "loading" with histidine monohydrochloride administered orally in an appropriate menstruum, and therapy with folic acid and other medications are described in the discussion.

RESULTS

Control Subjects

Urinary excretion of FIGLU in normal subjects, determined by the enzyme assay, ranged from 0 to 10 μg/ml with an average of 0 to 0.8 μg/ml; and from 0 to 5 mg per 24 hours, with an average of 0.6 mg per 24 hours.

H/U ratios by the *L. arabinosus* assay ranged from 0.1 to 0.6, with an average of 0.2 to 0.4.

In our experience, FIGLU values in urine above 30 μg/ml by the enzyme assay, or an H/U ratio above 0.9 by the microbiologic assay, represent significantly increased excretion of FIGLU.

TABLE II

Urine Content of FIGLU in Macrocytic Anemias

Patient	Sex and Age (yr.)	Type of Anemia	Urine Content of FIGLU μg/ml
B. B.	F, 45	Nutritional macrocytic anemia	60
T. P.	M, 49	Nutritional macrocytic anemia	275
J. G.	M, 54	Nutritional macrocytic anemia	120
J. H.	F, 32	Macrocytic anemia of pregnancy	87
E. C.	F, 26	Macrocytic anemia of pregnancy	64
J. R.	F, 3	Megaloblastic anemia of infancy	261

FIGLU in Megaloblastic Macrocytic Anemia

A number of patients with severe megaloblastic macrocytic anemia were found to have 60 to 275 μg FIGLU per ml of urine, as illustrated in Table II. The majority of such patients, however, had urinary FIGLU concentrations within normal limits. In our experience with 14 patients having addisonian pernicious anemia in relapse, and 47 patients with other types of nonmegaloblastic anemias, we found no increase in urinary FIGLU. In patients with an increase of FIGLU in the urine, administration of therapeutic doses of folic acid invariably resulted in a reduction of the FIGLU content to normal levels within 48 hours. Administration of vitamin B_{12} had no comparable effect.

FIGLU Following Histidine Metabolic Loading in Megaloblastic Macrocytic Anemia

Because many patients with megaloblastic anemias, apparently due to folic acid deficiency, did not have above normal amounts of FIGLU in their urine, we determined whether or not the FIGLU content in the urine could be influenced by administering histidine, the normal amino acid precursor of FIGLU. If the patient's folic acid activity was reduced, the increased FIGLU formed from the additional histidine could not be completely metabolized and would be excreted in the urine. Under the conditions of our histidine "metabolic loading" procedure, FIGLU excretion in urine was invariably increased, by the enzyme assay, in patients with nutritional macrocytic anemia megaloblastic macrocytic anemia associated with sprue, malabsorption syndromes, pregnancy, infancy, and liver disease. Patients with "pure" addisonian pernicious anemia as

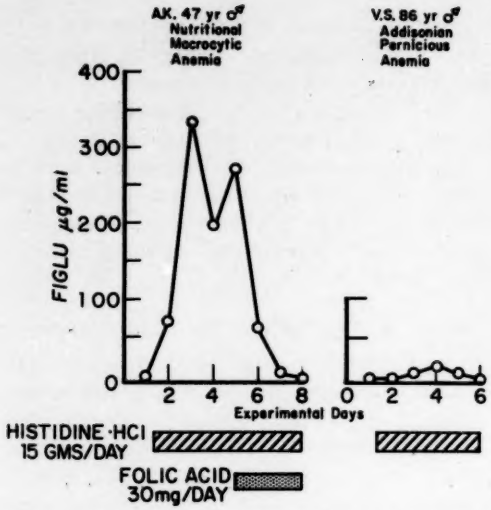

Fig. 2. Urinary excretion of FIGLU during oral histidine metabolic loading in patient with nutritional macrocytic anemia compared with that in patient with addisonian pernicious anemia in relapse.

well as normal subjects, showed no significantly increased amounts of FIGLU in the urine under the conditions of this test.

Examples of results with histidine metabolic loading are shown in Figures 2, 3, and 4.

The urinary excretion of FIGLU after histidine metabolic loading in a case of nutritional macrocytic anemia is compared with that in addisonian pernicious anemia in Figure 2.

Patient A. K., a 47-year-old male with nutritional macrocytic anemia, entered the hospital with the chief complaint of progressive weakness of two months' duration. Major physical findings were moderate emaciation, pallor, and smooth tongue. Neurologic examination was within normal limits. Laboratory findings revealed a hemoglobin of 6.6 g per 100 ml; mean corpuscular volume (MCV) of 105 cu μ; and a megaloblastic bone marrow pattern. Gastric acidity was normal; serum vitamin B_{12} concentration was 700 $\mu\mu$g/ml; and a bone marrow stain for iron was normal.

The initial amount of FIGLU in the urine was 4.2 μg/ml as determined by enzyme assay. Within 48 hours after commencing oral histidine metabolic loading at 15 g per day, the amount of FIGLU in the urine reached 350 μg/ml. Administration of folic acid produced a dramatic decrease within 24 hours in the FIGLU content in the urine which reached normal values the next day.

Patient V. S., an 86-year-old male with addisonian pernicious anemia, entered the hospital with the chief complaint of progressive weakness, and increasing difficulty in walking of three months' duration. Major physical findings were emaciation, pallor, and glossitis. Neurologic examination revealed impaired vibratory sense and a positive Romberg sign. Laboratory examination revealed a hemoglobin of 6.5 g per 100 ml; mean corpuscular volume of 108 cu μ; and a megaloblastic bone marrow pattern. There was a histamine-refractory gastric achlorhydria. Serum vitamin B_{12} concentration was 163 $\mu\mu$g/ml. Gastrointestinal absorption of vitamin $Co^{60}B_{12}$ by the Schilling test, performed after the histidine metabolic-loading studies to be described, was markedly decreased, but improved strikingly following the oral administration of $Co^{60}B_{12}$ with intrinsic factor. Dramatic hematologic improvement resulted from the parenterally administered vitamin B_{12}.

The initial FIGLU content in the urine was 0.32 μg/ml. After an oral histidine metabolic load similar to that given patient A. K., the greatest urinary excretion of FIGLU was 23.8 μg/ml.

The urinary excretion of FIGLU after histidine metabolic loading in a case of macrocytic anemia of pregnancy is compared with that in a patient with iron deficiency anemia of pregnancy in Figure 3.

Patient I. W., a 29-year-old female with macrocytic anemia of pregnancy, para IX gravida XI, entered the hospital at 30 weeks' gestation because of anemia. Physical findings were within normal limits. Laboratory examination revealed a hemoglobin of 7.9 g per 100 ml; a mean corpuscular volume of 101 cu μ. Bone marrow examination showed mild megaloblastic morphologic changes. Serum vitamin B_{12} concentration was 130 $\mu\mu$g/ml. No hematologic response was made to a 10-day course of 50 μg vitamin B_{12} intramuscularly daily. A slight improvement in the hemoglobin from 7.8 to 9 g per 100 ml followed two weeks of intravenous iron therapy totaling 1500 mg. At 37 weeks' gestation her hemoglobin was 9.3 g/100 ml; white blood cells, 5000 per cu mm; platelets, 80,000 per cu mm; bone marrow, still slightly megaloblastic.

The initial FIGLU content in the urine was 4.0 μg/ml by enzyme assay; the H/U ratio was 0.29 by *L. arabinosus* assay. Following oral histidine metabolic loading at 20 g per day, the FIGLU content in the urine was 120 μg/ml at 48 hours, 474 μg/ml at 72 hours, and reached a peak of 750 μg/ml on the eighth day. The H/U ratio at 48 hours was 1.6; on the eighth day, 4.1. Administration of a large oral dose of folic acid promptly lowered the elevated FIGLU content in urine to normal values within 24 hours, and complete hematologic response ensued.

Patient M. O., a 26-year-old female (para VI gravida VII) with iron deficiency anemia of pregnancy had a hemoglobin of 9.3 g/100 ml, and a normal bone marrow morphologic pattern.

The initial FIGLU content in urine was less than

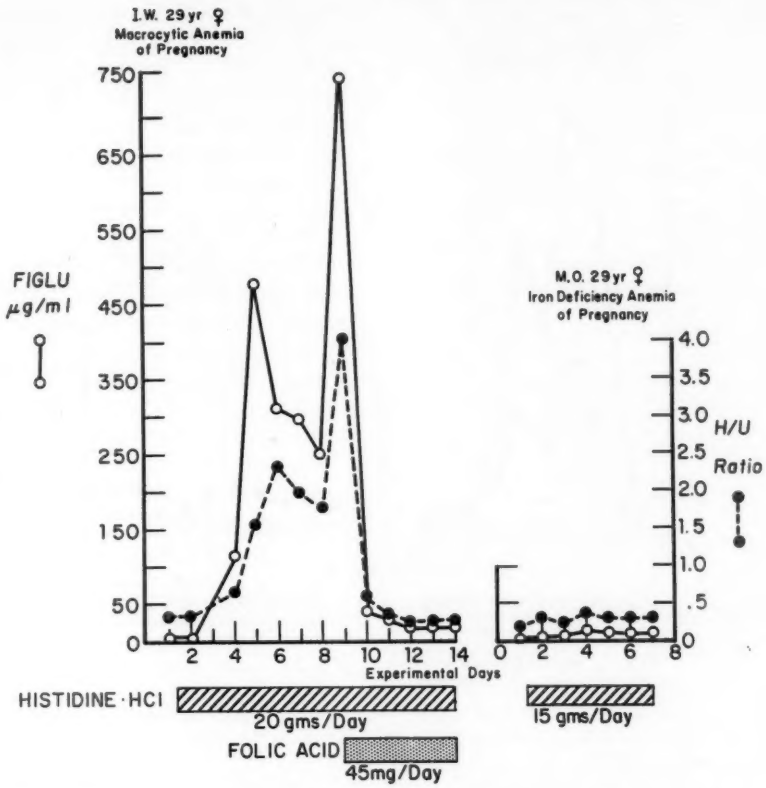

Fig. 3. Urinary excretion of FIGLU during oral histidine metabolic loading in patient with macrocytic anemia of pregnancy compared with that in patient with iron deficiency anemia of pregnancy.

0.1 μg/ml by enzyme assay; the H/U ratio 0.2. The patient was given an oral histidine metabolic load of 15 g daily. After six days of metabolic loading the greatest urinary excretion of FIGLU was 18.1 μg/ml and the highest H/U ratio 0.31.

The urinary excretion of FIGLU after histidine metabolic loading in a patient with macrocytic anemia associated with malabsorption syndrome, compared with that of a normal control, is illustrated in Figure 4.

L. S., a 70-year-old female with macrocytic anemia associated with malabsorption syndrome, entered the hospital with the chief complaints of diarrhea of six months' duration, weakness and anorexia of two months' duration. The major physical findings were severe emaciation and glossitis. Neurologic examination was within normal limits. Laboratory examination revealed a hemoglobin of 3.7 g/100 ml; a mean corpuscular volume of 104 cu μ; gastric free acid of 90 units after histamine; no gastric atrophy by biopsy; a serum vitamin B_{12} level of 77 μμg/ml. Gastrointestinal absorption of glucose was reduced; that of Co^{60} vitamin B_{12} by the Glass test was nil. Absorption following Co^{60} vitamin B_{12} with intrinsic factor was only 50 per cent of normal.

The initial FIGLU content in urine was 3.2 μg/ml by the enzyme assay; the H/U ratio 0.39. Following oral histidine metabolic loading of 12 g per day, the FIGLU content in urine was 490 μg ml at 48 hours, reaching a peak of 530 μg by the third day. The H/U ratio at 48 hours was 1.6; at the third day 2.1. Oral administration of folic acid lowered the elevated urinary excretion of FIGLU to normal values within 36 hours. A good hematologic response ensued.

Patient F. F., a 70-year-old male, who served as the normal control subject, had been hospitalized for a laceration of the left arm. He had no significant physical or laboratory abnormalities.

The initial FIGLU content in urine was less than 0.1 μg/ml; H/U ratio, 0.30. An oral histidine metabolic load of 20 g daily was given for ten days. At no time

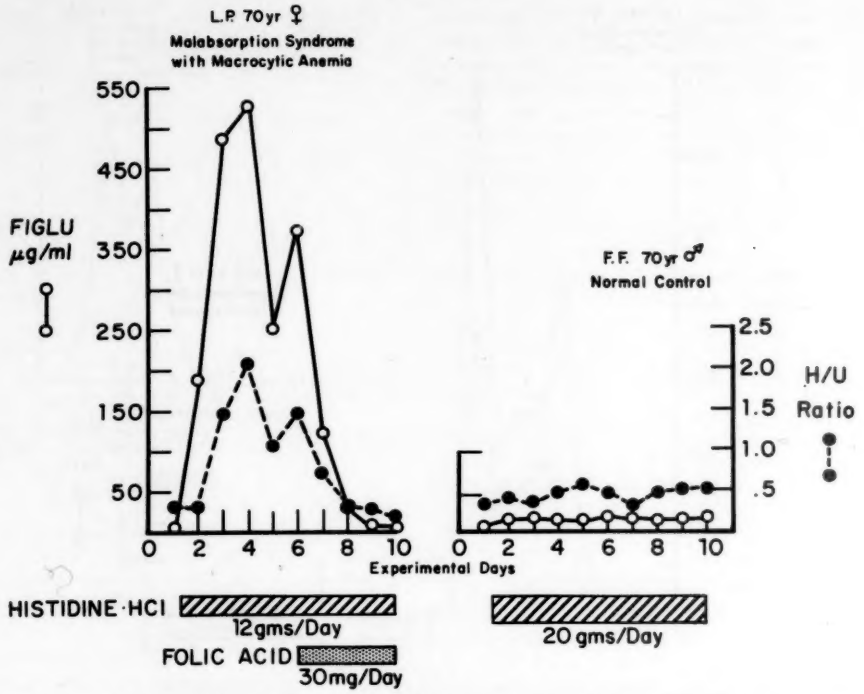

Fig. 4. Urinary excretion of FIGLU during oral histidine metabolic loading in patient with macrocytic anemia associated with gastrointestinal malabsorption syndrome compared with that in normal control subject.

was the urinary excretion of FIGLU above 20 μg/ml or the H/U ratio above 0.55.

DISCUSSION

In our experience to date with oral histidine metabolic loading in patients with various types of nonpernicious megaloblastic anemias, significantly increased urinary excretion of FIGLU always occurred within 48 hours after a 15 g daily dose of histidine monohydrochloride. In many of these patients, whenever it was possible to perform a therapeutic trial, the parenteral administration of vitamin B_{12} in doses of 10 to 100 μg resulted in incomplete or no hematologic response. On the other hand, the administration of small doses of folic acid produced dramatic hematologic and clinical improvement. Such data supports the contention that in these patients the primary deficiency was that of folic acid activity.

In ten patients with addisonian pernicious anemia, no significantly increased excretion of FIGLU could be induced with these doses given for as long as ten days. This indicates that in "pure" addisonian pernicious anemia folic acid activity does not differ from that in normal subjects under the metabolic conditions of the test. If such patients excrete increased FIGLU in the urine under histidine "loading," this indicates a combined vitamin B_{12} and folic acid deficiency. In normal subjects, no increased urinary excretion of FIGLU occurred under metabolic loading unless at least 36 to 45 g of histidine monohydrochloride was fed daily for several days.

Increased urinary excretion of FIGLU was also found under loading conditions in mild cases of borderline megaloblastic anemia, in which the morphologic evidence for megaloblastosis was equivocal. The test will therefore also indicate subclinical or premegaloblastic anemia folic acid insufficiency.

Our clinical experience with urinary excretion of FIGLU following the 15 g metabolic

loading procedure in patients with addisonian pernicious anemia is in contrast to the findings of Silverman and Pitney.[32] These authors reported increased FIGLU without histidine loading in the urine of patients with pernicious anemia assayed by their combined enzyme-microbiologic assay. Since no excretion figures for FIGLU were given, it is difficult to assess these findings. Although they also report finding increased urinary excretion of FIGLU in rats made vitamin B_{12}-deficient, our experience with vitamin B_{12} deficiency *in man* indicates that urinary excretion of FIGLU following our standard histidine metabolic load differentiates clinical disorders due to deficiency in folic acid metabolism from those of vitamin B_{12} deficiency.

SUMMARY

Folic acid deficiency results in inability to degrade a formiminoglutamic acid (FIGLU) to glutamic acid so that FIGLU accumulates in excessive amounts and is excreted in the urine.

The assay technics which have been developed for detection of FIGLU are described and evaluated.

Increased urinary FIGLU following an oral histidine monohydrochloride metabolic load of 15 g daily for two to three days is a biochemical index of folic acid deficiency. It is proposed as a new test to differentiate clinical folic acid deficiency states from those due to deficiency of vitamin B_{12}, such as addisonian pernicious anemia.

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Some Microbiologic Prospects for Discerning Vitamin Interrelationships

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Harry Sobotka, ph.d.,§ and S. Aaronson, ph.d.||

Vitamin interrelationships underlie much of a problem once posed in this Journal: "Today the nutrients essential for man appear to have been delineated. Yet how they interact and how their reduction or lack precisely affect the organism so as to give rise to specific clinical and pathologic changes which comprise human deficiency disease states are questions which have yet to be solved."[1]

Many of the answers must come from microbiologic enterprises. As we will show here, use of particle-ingesting (phagotrophic) protozoa opens to attack some problems which would be intimidatingly expensive to pursue solely with higher animals, not to mention with human patients.

PHAGOTROPHIC PROTOZOA

"True" protozoa are animals; that is, they eat. An omnivorous protozoon necessarily has digestive enzymes equivalent to a higher animal's, perhaps with more versatility in digesting microbial bodies. Several such protozoa can be grown in chemically defined media. Which most resembles man? To appreciate the practical import of this question, contrast the cost of a few racks of test tubes and roomfuls of rats or chicks for the equivalent experiment.

Two kinds of phagotrophs in pure culture are represented by the ciliate *Tetrahymena pyriformis* and yellow-brown photosynthetic chrysomonad flagellates such as *Ochromonas malhamensis* and *Poteriochromonas stipitata*. Other protozoa are in pure culture, e.g., small amebae and *Paramecium*, but this discussion is restricted to *Tetrahymena* and *Ochromonas*.

(1) *Tetrahymena: Possibilities in Its Amino Acid, Biotin, Vitamin B_6, and Folic Acid Requirements*

T. pyriformis, unlike man, requires neither fat-soluble factors (aside from the sterol requirement of a few strains) nor vitamin B_{12}, but it does require exogenous purine (primarily guanine) and thioctic (lipoic) acid. Its vitamin B_6 requirement cannot so far be bypassed by the amino acids (including D-alanine) effective for such lactic acid bacteria as *Streptococcus faecalis*.[2,3] We are trying to see whether feeding heat-killed *Str. faecalis* grown on a vitamin B_6-free medium to *Tetrahymena* supplemented with likely vitamin B_6-bypassing compounds, will bypass *Tetrahymena's* requirement. It would not be surprising if effective sparing, let alone bypassing, should demand a much longer roster of nitrogenous compounds than hitherto assembled: Vitamin B_6 enters

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into every known reaction having to do with amino acid or with moving nitrogen in and out of heterocycles. This makes the ease in bypassing the vitamin B_6-requirement of *Str. faecalis* hard to interpret. The present inability to bypass the vitamin B_6 requirement of *Tetrahymena* implies that when *Tetrahymena* serves as an assay organism for vitamin B_6, it is responding mainly to this nutrient, not to products of its catalytic activity. This implies further that the vitamin B_6 requirement of man may be spared by products of vitamin B_6 activity, and that such sparing should be taken into account in surveying foods for vitamin B_6 or in assaying vitamin B_6 in body fluids.

We are now studying the practicability of such assays with *Tetrahymena*. The problem of the vitamin B_6 requirement of *Tetrahymena* can be attacked by simultaneously feeding *Tetrahymena* an assortment of organisms whose vitamin B_6 requirement can be bypassed. The nonphagotrophic flagellate *Crithidia fasciculata* has a vitamin B_6 requirement that we have spared but not bypassed with a mixture of amino acids.[2] Which organism's vitamin B_6 requirement is most akin to man's is a problem to be solved in the future.

We may suppose that to bypass the vitamin B_6 requirement of both *Tetrahymena* and *Crithidia* one has to provide D-amino acids, as when vitamin B_6 is bypassed in vitamin B_6-requiring bacteria. Such a finding would heighten the likelihood that D-amino acids are essential metabolites for higher animals; it would make more attractive vitamin B_6-bypassing experiments with higher animals. To induce sharp vitamin B_6 deficiencies, it would probably be necessary to add a vitamin B_6-antagonist to the diet. We have not found a satisfactory antagonist for vitamin B_6 in protozoa; isonicotinic hydrazide holds some promise.

One can lead a phagotroph to a particle, but will it ingest? How could one tell whether negative results in bypassing experiments with phagotrophs reflect a failure of ingestion of particulate food, e.g., bacterial bodies or, if it is ingested, a failure to digest? One way might be to see whether *Tetrahymena* can satisfy one or more of its amino acid requirements from the particulate food in question. *T. pyriformis* has much the same pattern of amino acid requirements as man; it has been proposed—with convincing assay data—as a replacement for higher animals in measuring the biological value of proteins.[4]

Another tough problem is posed by the folic acid requirement of *Tetrahymena*. Here folic acid is spared, not bypassed, by thymidine plus the other metabolites effective for such folic-assay bacteria as *Str. faecalis* and *Lactobacillus casei*.[5] If the requirement for folic acid in man follows the *Tetrahymena* pattern, as seems likely, then *Tetrahymena* is the best organism for measuring the folic acid activity of foods and body fluids. In some experiments we supplied thymidine and other folic products as folic-sparers together with bodies of *Str. faecalis* grown on a folic-free medium, i.e., on a medium in which folic acid was wholly replaced with purines, pyrimidines, and amino acids. This bacterial supplement seemed to have little if any effect on *Tetrahymena* beyond that given by thymidine. The next move might be to supplement the *Str. faecalis* bodies with bodies of *C. fasciculata* grown on a folic-free medium.

To bypass the folic acid requirement of *Crithidia*, the new pteridine growth factor, biopterin, had to be supplied.[2] This brings up a new problem: can biopterin by bypassed? Bypassing folic acid for *Crithidia* thus resembles the Quaker Oats box whose label shows a Quaker holding a box whose label shows a Quaker, etc. Since no other biopterin-requiring organism is known, *Crithidia* cannot be given a crude (i.e., an unfractionated natural product) rendered biopterin-free by the device of bypassing the biopterin requirement. Another course is to inactivate the biopterin in a crude by illuminating the preparation: biopterin, like folic acid, is light-sensitive. Retention of *Crithidia*-factor activity beyond the inactivation of an added control amount of biopterin would point either to new bypassing compounds or to unfamiliar forms of *Crithidia* factor. Obviously the same procedure could be followed directly in bypassing the folic acid requirement of *Tetrahymena* or, for that matter, higher animals. Here too we meet the Quaker Oats or Chinese box situation: to spare folic acid in many bacteria, vitamin B_{12} is needed. Can this

vitamin B_{12} requirement be bypassed in turn?

(2) *Ochromonas malhamensis*

Since *Ochromonas* has a vitamin B_{12} requirement seemingly identical with man's, the *Ochromonas* method is now the official British method of measuring vitamin B_{12}; rats and chicks here became technologically unemployed. *Ochromonas*'s requirement for the vitamin has no more been bypassed than has man's. The implication is that vitamin B_{12} helps synthesize unknown compounds. In preliminary experiments, *Ochromonas* was fed *Escherichia coli* 113-3 grown on methionine rather than vitamin B_{12} and, separately, on *L. leichmannii* grown on thymidine instead of vitamin B_{12}. *Ochromonas* still needed vitamin B_{12}. This may mean that: (1) the bacteria were practically free of vitamin B_{12}—their vitamin B_{12} requirement may have been bypassed; (2) *Ochromonas* uses vitamin B_{12} for syntheses absent in bacteria.

Ochromonas has no clear-cut requirements for amino acids. To tell whether particulate food is being effectively utilized, one might exploit instead its biotin and thiamine requirements: If *Ochromonas* can get both vitamins from particulate food, presumably the particles are being effectively utilized. Assays for thiamine and biotin by means of *Ochromonas* are likely to yield realistic parallels for higher-animal activity.[6] *Tetrahymena* also requires biotin and thiamine, which provides a unique cross-check—one kind of animal against another. *Tetrahymena* and *Ochromonas* are on different evolutionary lines; they are far more different from each other than the rat is from the chick.

Pernicious anemia exemplifies an interrelationship, a reciprocal mobilization, between two vitamins, B_{12} and folic acid. Microbiologic data, marshalled in a review in preparation, lead one to think that folic acid is necessary for the synthesis of at least three different parts of the vitamin B_{12} molecule; further, that folic acid joins with vitamin B_{12} in the synthesis of (1) itself; (2) methionine; (3) purines; and (4) thymidine. Folic acid (or folic-catalyzed products) seem necessary in turn for its own synthesis. The academic biochemist must be thankful for the existence of pernicious anemia, and hence to clinicians, for calling attention to vitamin B_{12}. The yet obscure folic acid-vitamin B_{12} link in pernicious anemia stands as a sign post to a web of reaction pathways controlled importantly by multiple feedbacks. Awareness of catalysts which, amid their other activities, apparently catalyze their own synthesis, raises the question of where the controls on growth, conventionally ascribed to the nucleic acids, enter. Tracing vitamin interrelationships inevitably leads to deep waters: What limits the autonomy of "self-duplicating" intracellular systems? What is the significance of "priming" or "sparking" reactions—whence the original primers? And, more broadly, how is metabolism and growth coordinated at the molecular level?

PROSPECTS AND CONCLUSIONS

T. H. Jukes says, "nothing is cheaper than an idea." How easy it is to draft grandiose research projects! We plead in extenuation that we have confined our speculations to experiments actually in progress or plainly being designed. Nonetheless some experiments are so complicated that pilot experiments with microorganisms seem the prudent course before experimenting along these lines with higher animals. Some work with protozoa, such as on "temperature factors," designed to find out why the requirement for vitamin B_{12} in animals is increased sharply in hyperthyroidism, is probably at the present limits of practicability with higher animals.[7,8] We must hope that the protozoa used do have a metabolism like ours—that, to put it not all together facetiously, they are humanoid. Our present candidate is *O. malhamensis*. The limiting factor in reaching a decision may turn out to be the slowness of parallel experiments with higher animals.[7] An eminent chemist, now dead, once asserted to one of us that the limiting factor in hematology had been the development of chemical methods for fractionating blood constituents, not the development of analytical methods. The rejoinder was that knowledge of vitamin B_{12} had not advanced rapidly when the analytical method consisted of a hospital ward of patients with pernicious anemia; once microbiological methods were aligned with clinical findings, then large-scale

chemical fractionation of vitamin B_{12}—at least on this side of the Atlantic—could proceed rapidly, through intermediate stages of rat and chick assays. This and many other examples attest that it is of the utmost importance to reduce the lags in the sequence microbe→higher laboratory animal→man. The choice of microbial research problems is shaped to some degree by a desire to complete the circle: man→microbe. Indeed it is important to reduce the lag in traversing this research circle in both directions.

The need to make microbiological assays as simple as possible for clinical use is often impressed upon us. Toward this end we have listed many pointers.[9]

Electrical engineers speak of the retina, the brain, or of the organism itself, as a "black box:" sensory signals enter, and appropriate behavior follows—how is the interpreting or transducing done in these black boxes? The pursuit of vitamin interrelationships, especially among vitamins having broad synthetic functions, such as those just discussed, will help pry the lid off the metabolic black box, and so, eventually, off the organismal black box—which is to say that we will have the insights into the reaction chains underlying nutritional requirements that clinicians, such as the one quoted at the opening of this discussion, are seeking.

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Effect of Pyridoxine and Pantothenic Acid Deficiency on Conditional Reflexes

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Thiamine and niacin in the diet have been recognized for many years as being necessary for life and normal mental function; their absence results in disease—e.g., beriberi, pellagra. However, the literature contains little direct evidence of such an effect of pantothenic acid or pyridoxine.

Our curiosity was aroused in regard to the possible effect on mental function of the deficiency of pantothenic acid and pyridoxine in 1940 when we became acquainted with the work of Wintrobe, who was then at the Johns Hopkins Hospital. Depriving young pigs under one year of age of these two vitamins, he noted various neurologic abnormalities—ataxia, degeneration in the spinal motor cells, as well as in the sensory neurons.[9-13] Anemia and blood alterations also occurred. These nervous symptoms and blood changes did not appear, however, until the deprivation had lasted for several months.

Would there be a corresponding impairment of higher nervous activity (at the basis of mental function)? How soon would it follow complete deprivation? Would it be reversible? The conditional reflex measures, being quantitative, seemed well suited to give answers to these questions.

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PROCEDURES

For this purpose we used two types of animals, rats and dogs; two kinds of inborn activities (unconditional reflexes), related to pain and to food; and we tested for both formation and retention of learned behavior (conditional reflexes). In the dogs we tested also for reversibility.

Four adult dogs were used, including one old one. These animals had been used in previous experiments for periods ranging from several months to three to four years, for the formation of stable conditional reflexes, both excitatory and inhibitory. Two tones were used, T256+ and T512−, the first given for ten seconds and immediately followed by faradic shock to the foot of sufficient intensity to cause withdrawal of the foot, and the second tone, given in random order and never reinforced. This procedure had been repeated so many times that the conditional reflexes could be considered as stable and well established. Moreover, the difference between two tones an octave apart is an easy one for the animal.

The dogs were switched to the stock diet containing all synthetic B group vitamins as control. Then the same diet was used with the omission of either pantothenic acid or pyridoxine. The vitamins were added to the diets in the form of capsules. The dogs were maintained on the deficient diet for several months, and then returned to the diet containing the vitamins formerly omitted. Conditional reflex records were taken for the two tones, according to the routine of repeating the two tones eight to ten times daily, five times weekly. This work was done in the early 1940s.

In the rats a different procedure was employed. Beginning this work in 1957, we

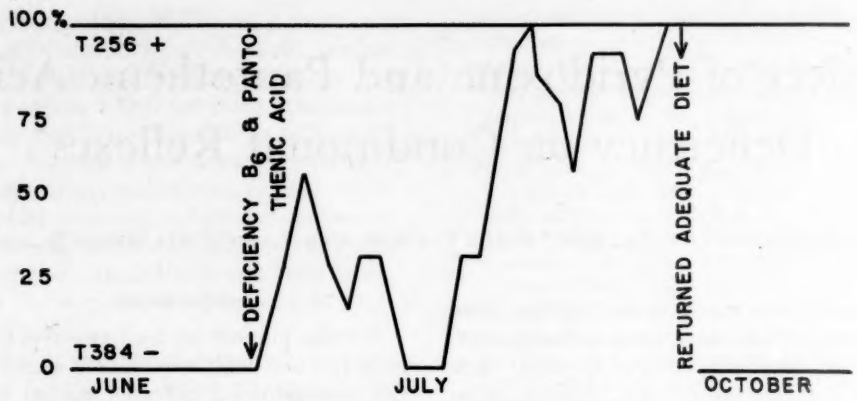

Fig. 1. Impairment differentiation caused by vitamin deficient diet. Impairment begins three days after deficiency.

divided six-week-old rats into three groups of five to ten each. Group I was placed on the adequate stock diet, containing the essential foodstuffs in natural form—milk, liver, yeast. corn oil, and salt. Group II had vitamin-free casein 29 per cent, sugar 60 per cent, corn oil 5 per cent, and salt 4 per cent, to which were added all known synthetic vitamins. The diet of group III was exactly the same as that of group II except that pyridoxine was omitted from the added vitamins.

Conditional reflex training in the rats, in contrast to the procedure used in the dogs, was not started until after the rats were placed on the diets. Furthermore the reinforcement was food instead of pain, and the conditional reflex used was running a maze to obtain food. Thus *formation* of conditional reflex was tested in the rats, and *retention* of conditional reflex in the dogs. The rats, starved 24 hours before experimentation, were run on a fairly simply elevated maze, without variation; i.e., the maze was not changed from day to day. Three grams of the same diet used for the respective group was the unconditional stimulus, placed at the end of the run. Each rat was given five runs daily. At the end of the run he was returned by hand to the starting point of the maze. His daily feeding was given immediately after his five runs.

The records taken were (1) time to make the run measured from the instant when the rat was placed on the maze and (2) the number of errors, i.e., the false turns taken in negotiating the maze. The rats were allowed water *ad lib.**

RESULTS

In the experiments with the dogs, impairment in differentiation began after 4 to 15 days' vitamin deprivation in animals which had shown no loss of differentiation between the two tones. This impairment was sometimes in the direction of failure on the excitatory side, sometimes on the inhibitory. Whether excitation or inhibition suffered was not due to the vitamins omitted but to the type of animal. (Type or "temperament," according to Pavlov, is a determining factor in how an individual will act under stress, i.e., whether by a predominance of excitation or inhibition.) The loss of differentiation was never complete; it became progressively worse for several weeks, and then leveled off for the three to four months' period during which it was continued (Figs. 1 and 2).

When the dogs were then returned to the adequate diet, conditional reflex differentiation was restored to normal. In two dogs a second repetition of deficiency and return to normal showed essentially the same result, i.e., the process was reversible.

In dogs, in contrast to rats, there were no

* This procedure differs somewhat from that of Dr. O. D. Murphree, to whom I am indebted for instruction in maze running.

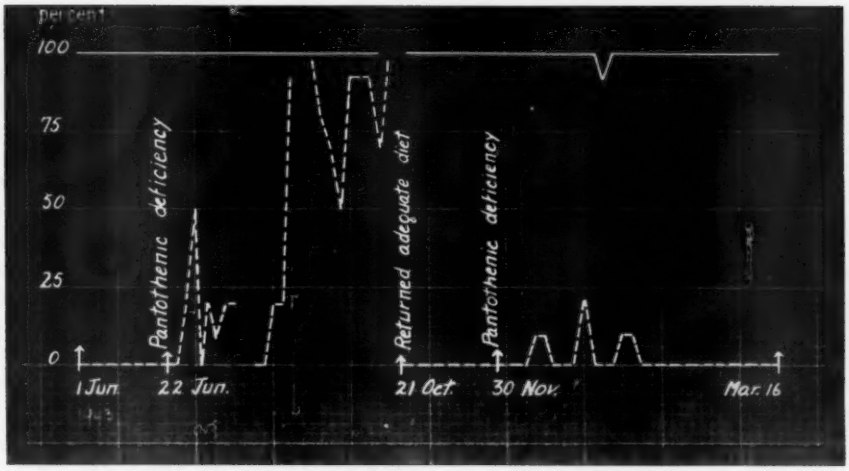

Fig. 2. Effect of vitamin deficiency on conditional reflexes. Note impairment of differentiation and return of differentiation with adequate diet.

other changes in behavior, and there was no alteration of reflexes—knee jerk, hopping, placing—or of artificial sexual reflexes.[4,5]

The dietary deprivation had a greater effect on the rats than on the dogs. There were gross behavioral alterations, marked physical changes, and in group III there was loss of weight and death.

The description of the behavior in the rats follows: group I, they were active, hungry, undisturbed by noises or people; group II, they were restless, nervous when handled, easily startled, increased thirst; group III, they were indifferent to food, squealing, attempting to escape, trembling, chattering teeth, stiff and motionless when handled, snuffling, startled by loud noises.

The details of the behavior in the rats were as follows: group I, these rats showed no fear of noises or people; group II, these rats trembled sometimes when picked up and some of the members did not seem to be as active as those in group I. The notes made on the rats in group III at the time of experimentation follow:

"These rats now range from complete apathy to biting and from motionless behavior on maze to dashing about the cage when replaced, and chattering, attempting to escape, squealing at times and batting heads against the top of the cage wire to push it up. When handled some will bite, then remain still and tremble, then excrete in experimenter's hand, something that the rats in the other two groups have not yet done. Their performance on the maze entails long periods of simply sitting on end of maze where originally placed and not moving for periods of five minutes and sometimes longer. When they do move it is only a small distance, then they wait.

If they run rapidly they make many errors. Generally their program is to wait a long time, for the first five to ten minutes, with errors at that time being fairly common, but when speed is picked up later in the running, they make many errors and run wildly up and down the maze, sometimes without any interest evinced in the food after they reach it, but they will run and go back and forth on the maze. They neglect food at times when fed in the cage but drink water. At times they curl up in a ball refusing to be handled or placed on the maze and will not run at all. They show much ruffling of their coats. There is a dingy hue to the fur, and the feet and nose seem quite red and irritated at times."

When the rats were demonstrated to visitors, there was sometimes difficulty in seeing a gross behavior or physical deficiency between the rats in group I and those in group II, but even untrained observers had no hesitancy in deciding that the rats in group III were abnormal and different from those in groups I and II.

The weights of animals in groups I and II were not markedly different, but those in group III showed a definite loss of weight. The figures on the average for August to March were in groups I and II, 675 g; in group III, 450 g. The running times (520 trials) were

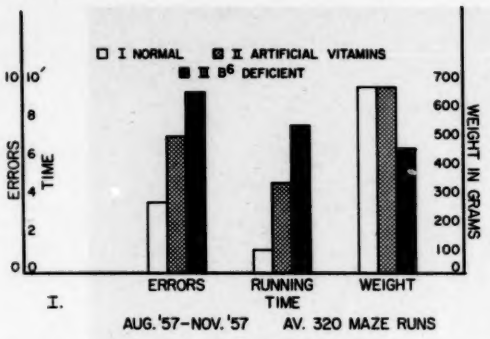

Fig. 3. Effect of vitamin deficiency on conditional reflexes: maze running rats (I) natural diet; (II) synthetic vitamin diet; (III) pyridoxine deficient synthetic diet.

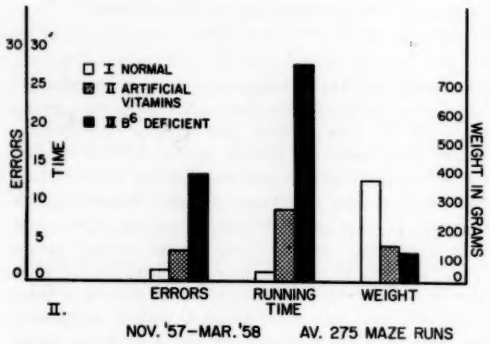

Fig. 4. Effects of vitamin deficiency on conditional reflexes: maze running in rats. (I) natural diet; (II) synthetic vitamin diet; (III) pyridoxine deficiency synthetic diet.

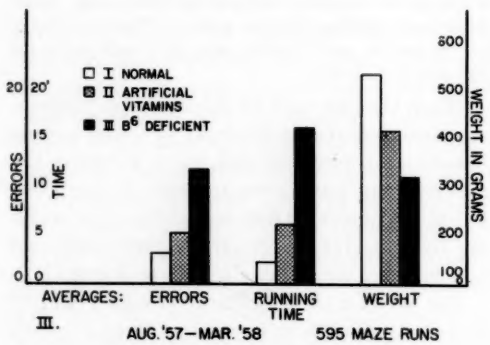

Fig. 5. Effect of vitamin deficiency on conditional reflexes: maze running in rats. (I) natural diet; (II) synthetic vitamin diet; (III) pyridoxine deficient synthetic diet.

group I, 1 minute 38 seconds with 3.8 errors; group II, 4 minutes 22 seconds with 6.9 errors; group III, 7 minutes 33 seconds with 9.8 errors (Fig. 3, 4, and 5).

DISCUSSION

Of the two species of mammal (dog and rat) tested for the effect of pantothenic acid or pyridoxine deficiency, the rats showed much greater disturbance in both general behavior and degree of conditional reflex disturbance. The rats, however, were young when the deficiency was started, while the dogs were adult. The dogs, moreover, did not show neurologic changes, while the rats did.

In the dogs, owing to the more accurate quantitative studies which had been carried out by one of us (W. H. G.), it is possible to equate the impairment of the conditional reflex with that of other agents, such as alcohol, anoxia, morphine. The vitamin deficiency produced as great a loss of the conditional reflex function as did either 2 cc alcohol/kg,[1] 18,000 feet altitude for four hours, $3^1/_2$ mg/kg morphine.[2,3,6–8] However, there were important differences: The alcohol in this dose produced marked ataxia, with return of function after the acute stage; the impairment from the anoxia continued for a year and was only slowly reversible; and morphine often causes ataxia. It is interesting that in the human subject suffering from chronic alcoholism in whom there is perhaps a vitamin deficiency, e.g., in Korsakov's psychosis, the conditional reflex loss is irreversible.[6]

We do not have an explanation of why the rats suffered more than the dogs, unless it be the age factor. In the rats there was no opportunity to eat their own feces, while in the dogs this was not controlled.

However, both these experiments indicate that whether pain or food is used as the unconditional stimulus, whether the subject tested is a dog or rat, whether the function tested is the ability to retain well-established conditional reflexes (dogs) or to form new conditional reflexes (rats), the impairment is marked.

The difference between the best natural diet and the best synthetic diet containing all

known added vitamins is clear in the rat experiments. There is thus some superiority of the natural diet over the synthetic diet, but the tremendous difference is between the best synthetic diet (group II) and the same diet with omission of pyridoxine.

The loss of conditional reflex function corresponds to features equivalent to or related to mental function in man.

CONCLUSIONS

Pantothenic acid or pyridoxine deficiency in dogs causes a loss of conditional reflex performance comparable to loss of mental function in man, e.g., in Korsakov's psychosis. This loss appears within four to ten days before any other neurologic symptom or blood alteration, and before or without observable changes in behavior. The effect is reversible; i.e., the conditional reflex function becomes normal when the animal is returned to the adequate diet.

In rats placed on (I) a natural adequate diet, (II) the best synthetic vitamin diet containing all *known* vitamins, and (III) the same diet as II with the omission of pyridoxine, the animals in group I did slightly better than those in group II, but there was a significant superiority of the animals in group II over those in group III.

Both *retention* of conditional reflexes (dogs) and the ability to *form* conditional reflexes (rats) suffered during pyridoxine deficiency. There was a loss of conditional reflex function with pyridoxine deficiency whether the conditional reflex was based on food or on pain.

The loss of conditional reflex function in the dogs by pyridoxine deficiency was equated to the loss resulting from a moderately large dose of alcohol or of morphine or of anoxia. Pyridoxine apparently is necessary in the diet not only for physical health, but for normal conditional reflex function, which is the basis of mental performance.

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Vitamin Interrelationships in Heme Synthesis

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NORMAL RED CELL formation involves a number of diversified processes, which include the biosynthesis of nucleic acids, porphyrin, heme, and proteins. Limitations placed on the synthesis of any of these could conceivably lead to an anemia. The hematologic characteristics[1] of specific anemias undoubtedly are determined largely by the specific limitations involved, and investigators have for some time been interested in finding a biochemical explanation for each of the vitamin deficiency anemias. For example, the macrocytic anemia resulting from folic acid deficiency is considered to be due to an impaired nucleic acid synthesis which results in an inability of bone marrow to synthesize new cells.[1,2] The microcytic anemia of vitamin B_6 deficiency has been compared to an iron deficiency anemia, in which the microcytic hypochromic characteristics of the cells reflect a limited capacity of cells for hemoglobin synthesis. Cartwright and Wintrobe[3] suggested that the anemia of vitamin B_6 deficiency resulted from a defect in protoporphyrin synthesis. Schulman and Richert[4] found evidence that pyridoxal phosphate‡ was involved in heme synthesis for the formation of δ-aminolevulinic acid (ALA) from glycine and succinate. However, the vitamin also appeared to be essential elsewhere in the process of red cell formation since the administration of ALA did not alleviate the anemia.

The moderate anemia in riboflavin deficiency has been described as being normocytic in swine[5] and hypochromic microcytic in dogs, with a decrease in the number of cells.[6] Spector *et al.*[6] speculated that riboflavin might be concerned in the metabolism of the amino acids of the protein portion of the hemoglobin molecule.

Rinehart *et al.*[7] found that in thiamine deficiency anemia in monkeys the reticulocyte count fell to zero in 40 days. They suggested the anemia was due to a suppression of reticulocyte formation. In pantothenic acid deficiency anemia without accompanying granulocytopenia, the marrow erythroid cell index was not significantly lowered in rats.[8] Carter *et al.*[9] observed a hypochromic anemia in this deficiency in rats and reported that the morphology was similar to that seen in vitamin B_6 deficiency described by Wintrobe.

Handler and Featherston[10] studied the nature of anemia in dogs fed a niacin deficient diet. In some animals it was macrocytic in character, and the normochromic nature made it unlikely that a failure of hemoglobin synthesis was involved. These investigators believed that the need of erythrocytes for nicotinic acid is a general phenomenon, and is required for the growth of all cells of the animal organism; a study of erythrocytes permitted these observations because of the rapid rate of turnover.

With the rapid advancements in the details of porphyrin biosynthesis, recently reviewed by Rimington,[11] it has become possible to study the relationship of vitamins to heme formation in red cells of peripheral blood.

The essential features of the scheme by which heme is now believed to be biosynthesized can

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‡ The following abbreviations are used: δ-Aminolevulinic acid, ALA; pyridoxal-5-phosphate, B_6PO_4; diphosphopyridine nucleotide, DPN.

be summarized as follows: The first step is the reaction of succinyl coenzyme A with glycine to form α-amino-β-keto-adipic acid which upon decarboxylation yields ALA.[12] Two molecules of ALA react through δ-aminolevulinic acid dehydrase[13] to form porphobilinogen. Porphobilinogen is converted enzymatically to uroporphyrinogen which in turn is decarboxylated to coproporphyrinogen.[14] The next step is the formation of protoporphyrin which Granick and Mauzerall[15] have confirmed with preparations from red cells and *Euglena*. Iron probably makes its entrance into the molecule at the protoporphyrin stage.[14,16]

The incorporation of radioactive glycine,[17] ALA,[12,18] and porphobilinogen[19] into heme can be studied conveniently by incubating avian red cells or cell hemolysates with the substrate, then isolating and counting the radioactive heme as crystalline hemin.

EXPERIMENTAL STUDIES

The purpose of these experimental studies was to see whether or not the rate of incorporation of different labeled precursors (glycine and δ-aminolevulinic acid) into heme by red blood cells obtained from ducks was affected by the following vitamin deficiencies: vitamin B_6, pantothenic acid, niacin, thiamine, and riboflavin. The rate of glycine uptake in all of these deficiencies was reduced 50 per cent or more as compared with the controls; the rate of ALA uptake was reduced by 25 per cent or less. On the surface it appeared that each of the vitamins was involved more in the incorporation of glycine than ALA. However, when isotope incorporation into heme of control blood was plotted against the reticulocyte count, a straight line relationship was found with both glycine and ALA precursors. Extrapolation of the glycine curve to zero reticulocyte concentration showed that essentially no glycine was incorporated into heme in the absence of reticulocytes. When a similar extrapolation of ALA versus reticulocytes was made, a significant amount of ALA was calculated to be incorporated in the absence of reticulocytes and therefore by mature cells. Increases beyond this minimal level were also due to reticulocytes. Hence, the total number of reticulocytes present in the blood was the major factor which determined the rate of incorporation of glycine or ALA into heme, and the decreased rate of incorporation found in these vitamin deficiencies was primarily due to a decrease in the proportion of reticulocytes in the cell population. The greater effect which appeared to be obtained with glycine as compared with ALA was due to the fact that incorporation of glycine was due entirely to reticulocytes, while that of ALA was due only in part to the reticulocytes.

METHODS

Day-old Peking ducklings were fed Park and Pollard chicken mash for one to seven days and then vitamin deficient or control diets as described by Hegsted and co-workers.[20–22] Deficiency symptoms, as judged by growth, usually appeared in three to five days. As pointed out by Hegsted, ducklings grow at a rapid rate, and the rapidity with which nutritional deficiencies can be obtained makes this a convenient species for the study of such deficiencies.

Details of the procedures for collecting the blood and for incubating radioactive glycine with whole duck blood and radioactive ALA with red cell hemolysates have been described;[4] in these studies the hemolysates were not supplemented with KCl and $MgCl_2$ but with ferrous ammonium sulfate (7μg of Fe/ml). In most of the studies reported here, 20 μ moles of glycine-2-C^{14} (18,500 cpm (counts per minute) per μ mole) and 2 μ moles of ALA-2,3-C^{14} (11,000 cpm per μ mole) were used in the incubations. The rates of incorporation of the precursors were determined by measuring the specific activities (counts per minute per mg) of the isolated crystalline hemin. Blood smears for reticulocyte counts were stained by the procedure of Manwell and Feigelson.[23]

RESULTS

Vitamin B_6 Deficiency

In a previous study[4] it was found that heme was synthesized in the red cells of vitamin B_6 deficient ducklings from glycine-2-C^{14} at a rate which was half, or less than half of that found in control ducklings; the addition of pyridoxal-

TABLE I

Effect of Duration of Vitamin B_6 Deficiency on Rate of Incorporation of Glycine and ALA into Heme and Its Stimulation by Pyridoxal Phosphate
(Values in counts per minute per mg of hemin)[a]

	5 Days on diet		10 Days on diet	
		Plus B_6-PO_4		Plus B_6-PO_4[b]
Control whole blood with glycine-2-C^{14}	594	597	775	785
Vitamin B_6-deficient whole blood with glycine-2-C^{14}	253	416	133	301
Control hemolysate with ALA-2,3-C^{14}	130	[c]	427	395
Vitamin B_6-deficient hemolysate with ALA-2,3-C^{14}	121	[c]	258	168

From Schulman, M. P. and Richert, D. A., *J. Biol. Chem.* 226: 181, 1957.

[a] The specific activities of the labeled glycine and ALA were 147,500 and 14,500 cpm/μmole, respectively.

[b] 0.3 mg of pyridoxal phosphate monohydrate was added to each vessel containing 2 ml of duck blood. The total incubation volume was 2.3 ml.

[c] In other experiments the addition of pyridoxal-5-phosphate did not increase the incorporation of ALA.

5-phosphate *in vitro* stimulated the ability of the deficient cells to synthesize heme (Table I). Incorporation of ALA-2,3-C^{14} was affected less by the deficiency, and the addition of pyridoxal-5-phosphate (B_6-PO_4) did not stimulate it. The blood samples were less able to incorporate glycine or ALA into heme with increasing duration of the deficiency period, but B_6-PO_4 was still able to stimulate heme synthesis from glycine.

To test whether the anemia seen in vitamin B_6 deficient ducks was due solely to a retarded synthesis of ALA, ducklings were injected subcutaneously with ALA during the last 7 of the 11 days in which they were maintained on the deficient diet. Hemoglobin levels for the control, the deficient, and the ALA-injected deficient ducklings were 9.4, 6.7, and 6.1 per cent, respectively. The administration of ALA did not correct the anemia, and it appeared that vitamin B_6 must also be essential elsewhere in the process of red cell formation. From these results it was concluded that pyridoxal phosphate was necessary in heme synthesis for the conversion of glycine to ALA. This has been substantiated recently by other investigators who have demonstrated the requirement of this cofactor in the direct synthesis of ALA by fractions from chicken erythrocytes,[24–26] and by extracts of *Rhodopseudomonas spheroides*.[27,28]

Pantothenic Acid Deficiency

In view of Shemin's suggestion[12] that glycine reacts with succinate in the form of succinyl coenzyme A, the effect of pantothenic acid deficiency on heme synthesis was also studied.[4] It was found that the synthesis of heme from glycine and succinate was retarded by the pantothenic acid deficiency; greater effects were observed as the deficiency was prolonged. The uptake of ALA was also depressed; this was surprising since coenzyme A has been implicated solely for ALA synthesis in the process of heme formation. Therefore, it was necessary to look for another explanation for the effect of this and perhaps other deficiencies on heme synthesis.

There have been reports[29–31] that the process of porphyrin and heme synthesis is more active in immature cells than in mature cells. This suggested that the young red cell population (reticulocytes) might have been diminished in the vitamin deficiencies and that this in turn suppressed heme synthesis. Table II shows the effects of vitamin B_6 and pantothenic acid deficiencies on the reticulocytes and on the incorporation of glycine-2-C^{14} into heme. As the deficiencies progressed, both the incorporation of glycine and the reticulocyte counts decreased. After the five-day vitamin B_6 deficiency period, the reticulocyte count was 9.8 per cent, while the control count was 13 per cent. After ten days it had fallen to about 1 per cent. The hemin activities of the deficient bloods after five and ten days were 80 and 25 cpm, respectively. It was more difficult to restore the rate of glycine incorporation with B_6-PO_4 as the deficiency progressed.

Bloods from ducklings on the diet deficient in pantothenic acid for four days had 8.3 per cent reticulocytes, and after seven days only 1.5 per cent. Hemin activities were 87 and 27

TABLE II

Effects of Duration of Vitamin B_6- and Pantothenic Acid Deficiencies on Reticulocytes and on Incorporation of Glycine-2-C^{14} into Heme

	No. ducks	Wt. (g)	Hb (%)	Reticulocytes (%)	cpm per mg hemin
5 days B_6-deficient	8	79	8.2	9.8	80 ± 16.0
5 days B_6-deficient + B_6PO_4 (*in vitro*)	—	—	—	—	142 ± 30.2
5 days control	6	86	9.1	13.0	178 ± 25.0
5 days control + B_6PO_4 (*in vitro*)	—	—	—	—	170 ± 21.0
10 days B_6-deficient	9	86	8.9	1.1	25 ± 2.5
10 days B_6-deficient + B_6PO_4 (*in vitro*)	—	—	—	—	50 ± 8.4
10 days control	6	203	10.6	12.0	130 ± 13.5
4 days P.A.-deficient[a]	6	159	—	8.3	87 ± 11.3
4 days P.A.-deficient + 5 mg (*in vivo*) Ca pantothenate	9	163	—	8.1	113 ± 14.4
4 days control	8	195	—	13.4	194 ± 20.0
7 days P.A.-deficient[a]	6	123	—	1.5	27 ± 4.4
7 days P.A.-deficient + 5 mg (*in vivo*) Ca pantothenate	6	109	—	2.1	32 ± 3.2
7 days control	6	249	—	14.3	166 ± 9.8

[a] These were separate experiments done at different times. In the B_6-deficient and the pantothenic acid deficient experiment, the ducklings were on mash for one and four days, respectively, before feeding the synthetic diets.

cpm, respectively, compared with control values of 194 and 166 cpm.

The effect of injecting calcium pantothenate one hour before sacrifice on restoration of the rate of glycine uptake has been variable. The slight stimulation shown in Table II was not statistically significant, and in two other experiments essentially no response was obtained. In the study reported previously,[4] the response was significant. Reasons for these discrepancies are not known. Coenzyme A is known to be a cofactor involved in the synthesis of ALA.[24–28]

Other Vitamin Deficiencies

The next experiments were designed to study the effects of niacin, riboflavin, and thiamine deficiencies on reticulocyte counts and on the utilization of radioactive glycine and ALA for heme synthesis by blood from ducks. The deficiency periods were six or seven days. The results are shown in Table III. The pattern of heme synthesis from the two substrates was similar to that found in the vitamin B_6 and pantothenic acid deficiencies. The glycine uptake was 27, 36, 51, and 48 per cent of the corresponding control values in the niacin, pantothenic acid, riboflavin, and thiamine deficiencies, respectively; ALA uptake was 74, 79, 92, and 75 per cent of control. The reticulocyte counts were low, ranging from 1.5 to 2.5 per cent.

TABLE III

Effect of Vitamin Deficiencies on Rates of Incorporation of Glycine and ALA into Heme and on Reticulocytes of Duck Blood

Deficiency	No. ducks	Wt. (g)	Days on diet		Reticulocytes (%)	cpm per mg hemin	
			Mash	Synthetic		Glycine	ALA
Niacin	7	131	6	6	1.5	34 ± 3.5	123 ± 9.5
Pantothenic acid	8	185	6	6	1.8	46 ± 4.8	131 ± 6.0
Control	7	315	6	6	7.3	128 ± 9.4	167 ± 6.3
Riboflavin	5	136	6	7	2.5	42 ± 4.4	149 ± 7.6
Thiamine	5	140	6	7	1.8	40 ± 2.0	122 ± 8.4
Control	8	186	6	7	7.8	83 ± 11.0	163 ± 8.3

TABLE IV

Correlation of Incorporation of Glycine and ALA into Heme with Reticulocyte Counts

Experiment	cpm/mg hemin								
	Original			Top fraction			Bottom fraction		
	Reticulocytes (%)	Glycine	ALA	Reticulocytes (%)	Glycine	ALA	Reticulocytes (%)	Glycine	ALA
1	3.4	58	—	6.4	68	—	2.4	30	—
2	6.0	94	—	10.8	140	—	4.0	56	—
3	8.1	150	218	9.0	170	259	6.2	109	187
4	12.0	161	262	19.6	252	289	9.7	114	200
5	15.0	60	114	26.0	108	191	11.0	45	95
6	15.3	102	194	26.2	184	325	11.0	76	129
7	21.1	189	257	34.8	363	406	9.6	108	142
Av. (experiments 1–7 with glycine)	11.5	116	—	19.0	183	—	7.7	77	—
Av. (experiments 3–7 with ALA)	14.3	—	209	23.1	—	294	9.5	—	150

NOTE: More radioactive protoporphyrin accumulated when ALA was incubated with hemolysate than when glycine was incubated with whole blood. Dresel and Falk[36] found that when hemin crystals were prepared by Fischer's method from a mixture containing protoporphyrin, the crystals contained some protoporphyrin which could not be removed by washing. According to our figures approximately half of the radioactivity measured in hemin crystals prepared after incubation of hemolysate with ALA was due to protoporphyrin. Recrystallization did not alter this.

Several possible explanations were considered: (1) all these vitamins were involved in the enzymatic synthesis of ALA (2) the two systems used (whole blood for glycine and red cell hemolysates for ALA) were sufficiently different so that a valid comparison of the rates of uptake could not be made between the two substrates; and (3) the vitamin deficiencies suppressed new cell formation or the release of young cells into the peripheral blood, and mature cells synthesized heme more efficiently from ALA than from glycine. The relationship between the number of reticulocytes in blood and heme synthesis from glycine and ALA was examined to test the latter possibility. Red cells from a single blood sample were separated by differential centrifugation,[29] into a top fraction enriched with reticulocytes and a bottom fraction containing fewer reticulocytes. It was then possible to compare the rates of incorporation of glycine and ALA into heme by three samples of cells derived from the same blood (the original and two centrifuged samples) with varying numbers of reticulocytes. The results are shown in Table IV and in Figures 1, 2, and 3.

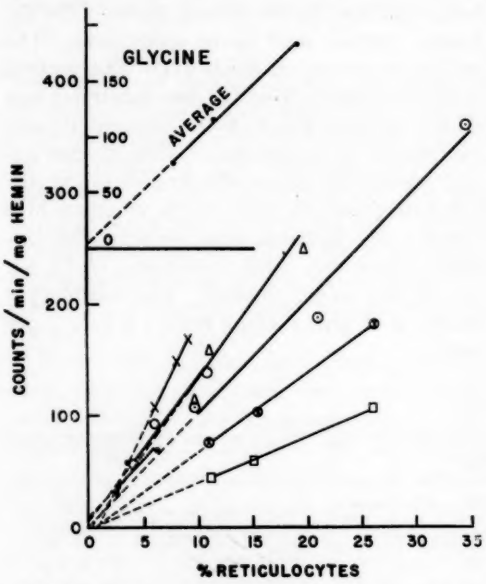

Fig. 1. Each line represents the reticulocyte count versus the incorporation of glycine for three samples of cells derived from the same blood by centrifugation (see text). The line in the upper figure is the average of the seven experiments.

In Figure 1, the percentage of reticulocytes (abscissa) was plotted against the specific activity of the hemin formed from radioactive glycine (ordinate) for each of the three samples derived from any given blood. This was done for all seven experiments. The three points usually fell on a fairly straight line (solid line) and each line passed very nearly through the

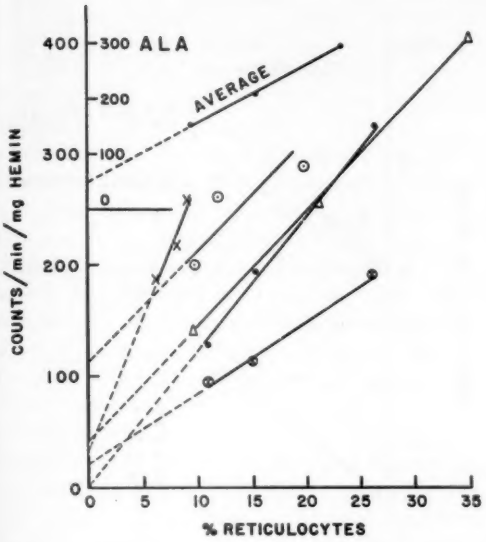

Fig. 2. Each line represents the reticulocyte count versus the incorporation of ALA for three samples of cell hemolysates derived from the same blood by centrifugation. The line in the upper figure is the average of the five experiments.

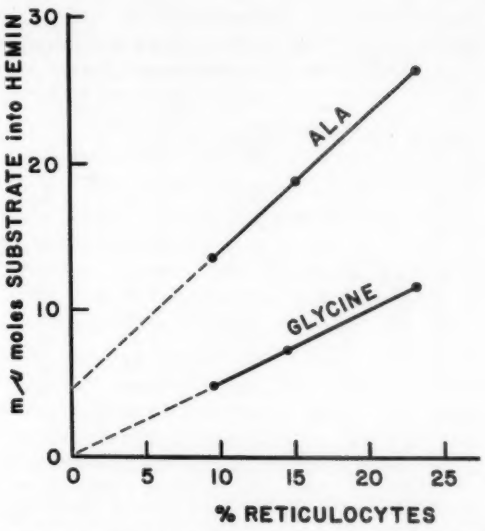

Fig. 3. Reticulocyte count versus mμ moles of glycine or ALA incorporation into heme. Each line represents the average values obtained from five experiments (see Table IV) in which both glycine and ALA were studied simultaneously.

origin when extrapolated to zero per cent reticulocytes. A line representing the average of the points (Table IV) is shown in the upper part of the figure.

The same thing was done in Figure 2 with the ALA data. There was considerable variability in different bloods, but the extrapolated lines clearly intersect somewhere above the origin. The average line from these five experiments intersected at approximately 50 cpm per mg hemin for zero per cent reticulocytes. This average figure cannot be used as a precise value for a given experiment because a different intercept was obtained for each sample of blood. But it does show that blood without reticulocytes would form small amounts of heme from ALA. The data also show that the incorporation of both glycine and ALA into heme is very dependent upon the reticulocytes.

The average mμ moles of glycine and of ALA incorporated into heme were calculated from the five experiments shown in Table IV in which both glycine and ALA were studied simultaneously. The points were plotted (Fig. 3) against reticulocytes and the lines were extrapolated to zero reticulocytes. The ALA line intersected at 4.5 mμ moles, while the glycine line passed through the origin.

The ratios of mμ moles of ALA:glycine incorporation into heme at various reticulocyte counts were calculated from Figure 3 and are shown in Table V. The corrected ratios were obtained after subtracting 4.5 mμ moles from each of the ALA values so that the contribution made by the mature cells was eliminated from the ALA data. These corrected ratios were constant for reticulocyte counts varying from 2 to 23 per cent. This shows that the rate of incorporation of both substrates increased in parallel and to the same degree with increasing reticulocyte counts. The uncorrected ratios do not give a true picture of the situation because the glycine line passes through the origin, while the ALA line intersects above the origin. This also explains why the incorporation of ALA into heme was decreased less by the vitamin deficiencies than was the incorporation of glycine.

In all the vitamin deficiencies studied, the incorporation of glycine and ALA was some-

TABLE V

Ratios of mμ Moles of ALA:Glycine Incorporation into Heme at Various Reticulocyte Levels

Reticulocytes (%)	mμ moles		Ratio ALA/glycine	
	ALA (1)	Glycine (2)	Uncorrected (1)/(2)	Corrected[a]
23	26.4	11.6	2.28	1.89
20	23.5	10.0	2.35	1.90
15	18.8	7.5	2.50	1.91
10	13.5	4.7	2.87	1.92
8	12.2	4.0	3.05	1.90
6	10.2	3.0	3.40	1.90
4	8.4	1.9	4.42	2.00
3	7.5	1.5	5.00	2.00
2	6.5	1.0	6.50	2.00
1	5.0	0.5	10.00	—

[a] The corrected ratio was obtained by subtracting 4.5 mμ moles from the ALA values in column (1) and dividing this value by the glycine value in column (2).

what higher than would have been obtained with control blood of the same reticulocyte count (on the basis of the appropriate extrapolated control line) (Fig. 4). Incorporation of glycine was higher by 44, 39, 61, and 100 per cent, and incorporation of ALA was higher by 66, 64, 75, and 59 per cent, respectively, in niacin, pantothenic acid, riboflavin, and thiamine deficiencies. If these incorporations are attributable to the reticulocytes present, then the deficient cells were not deficient in their ability to synthesize heme. On the other hand, it is possible that some of the cells in the vitamin deficient blood were characterized as mature by the staining technic, but still retained some reticulocyte characteristics and therefore a better ability to synthesize heme than control mature cells.

The question of whether the reticulocytes from deficient blood were more limited than control reticulocytes in the utilization of glycine as compared with ALA for heme synthesis cannot be clearly answered from these results. If it were assumed that (1) incorporation of ALA in the absence of reticulocytes remained constant at 50 cpm in all the experimental procedures and (2) the immature cells incorporated the remaining ALA and all the glycine linearly, then it would appear that the glycine uptake was more limited than the ALA

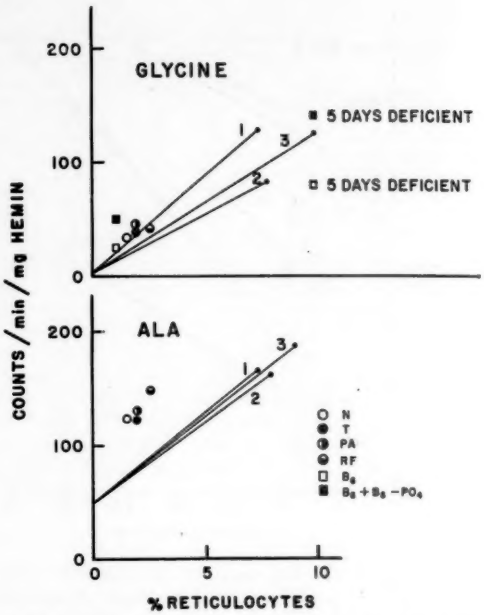

Fig. 4. Upper figure: Line 1 is a plot of the reticulocyte count (7.3 per cent) versus incorporation of glycine (128 cpm/mg hemin) for the control blood run simultaneously with the niacin and pantothenic acid deficiencies (see Table III). A line was drawn from this point through the origin. Line 2 represents the corresponding control value for the thiamine and riboflavin deficiencies. Line 3 represents the average of thirteen other experiments with control blood. Values for the blood samples taken during the various vitamin deficiencies are indicated by the symbols N = niacin; T = thiamine; PA = pantothenic acid; RF = riboflavin; B_6 = pyridoxine. The lower figure represents similar data for the reticulocyte count versus incorporation of ALA. Each line was drawn from the control point through the ordinate at 50 cpm/mg to correspond with the control value found in Figure 2.

uptake in the vitamin deficient reticulocytes. With the 50 count baseline subtracted from all the control and deficient ALA uptake values, incorporation of ALA was higher in deficient reticulocytes than in control reticulocytes by 204, 170, 183, and 166 per cent, respectively, in the niacin, pantothenic acid, riboflavin, and thiamine deficiencies. If these differences in incorporation of glycine and ALA by the vitamin deficient cells are real rather than artifactual, then the deficiencies studied would appear to have more effect on the incorporation of glycine into heme than of ALA.

DISCUSSION

Whether or not reticulocytes from deficient ducklings have a lowered capacity for utilization of glycine might better be demonstrated by measuring the glycine uptake in blood after a shorter term of deficiency and before the reticulocyte counts fall to very low levels. This was done in blood from ducklings deficient in vitamin B_6, and it can be seen in Figure 4 that the rate of glycine uptake was distinctly below that obtained with control blood with comparable reticulocyte counts after only five days of deficiency. The addition of B_6-PO_4 *in vitro* restored incorporation of glycine to within control values.

It is feasible that each of the vitamin deficiencies could hinder the synthesis of ALA in the red cells by different mechanisms. Granick[24] found that DPN in addition to glycine, B_6-PO_4, coenzyme A, glutamine, and inosine increased the rate of protoporphyrin synthesis by frozen and thawed hemolysates of red cells from chickens. He also emphasized the requirement of the citric acid cycle and an electron transfer system for the synthesis of ALA. Thiamine pyrophosphate, flavin adenine dinucleotide, coenzyme A, and DPN are coenzymes for these systems. While the requirements for B_6-PO_4, coenzyme A, and DPN in the synthesis of ALA from glycine have been demonstrated by *in vitro* studies,[24,26] the corresponding vitamin deficiencies (except for B_6) have not yet been shown to exhibit these specific biochemical lesions in heme synthesis; in the intact animal, the vitamin deficiencies appear to have a greater effect on the synthesis of new cells, indicated by low reticulocytes, rather than the production of normal numbers of defective cells.

A vitamin B_6 deficiency appeared to have two observable effects on the red cells of young ducklings: (1) The reticulocyte count was low, which in itself was responsible for decreased heme synthesis in the peripheral blood. (2) It limited heme synthesis from glycine by a mechanism which was independent of the decreased reticulocytes. The latter probably was due to a suppression of ALA synthesis, since pyridoxal phosphate is a required cofactor in the synthesis of ALA from glycine and succinate.[24,28] If the lowered reticulocytes reflect a decreased synthesis or release of red cells into the blood stream, this would account for a low cell count in this anemia.[32,33] In addition, a defect in the process of heme synthesis, which would put a limitation on the synthesis of a full complement of cell hemoglobin, could account for the small cells and their somewhat hypochromic nature.

If this interpretation is correct, then one might expect similar cell characteristics in pantothenic acid deficiency anemia. A similarity was reported by Carter *et al.*[9] Although pyridoxal phosphate and coenzyme A have widely differing functions in metabolism, and therefore might be expected to affect growth of cells for different reasons, their functions are also fused in a single reaction involved in hemoglobin synthesis in that both function as coenzymes in the synthesis of ALA.

Another important factor in considering the effects of vitamin deficiencies on heme synthesis is the possible effect on globin formation. Borsook[34] reported that the rates of synthesis of heme and of globin in rabbit reticulocytes *in vitro* seemed to be regulated so that both were formed at about the same rate. However, London and associates[35] found that the synthesis of heme and the incorporation of glycine into globin in avian red cells were readily dissociated in vitro by a variety of technics.

SUMMARY

The rates of incorporation of glycine-2-C^{14} and of δ-aminolevulinic acid-2,3-C^{14} into heme *in vitro* by erythrocytes of young control ducklings increased in a straight line relationship with the reticulocyte count when the isotope incorporation was plotted against reticulocyte count. Extrapolation of the glycine line to zero reticulocyte concentration showed that essentially no glycine was incorporated into heme in the absence of reticulocytes. However, a significant incorporation of δ-aminolevulinic acid took place in the absence of reticulocytes, but increases beyond this minimum value were also parallel to the reticulocyte count. The relatively higher incorporation of δ-aminolevulinic acid than of glycine into heme by blood which was very low in

reticulocytes could be attributed to this small utilization of ALA by mature cells.

The incorporation of radioactive glycine and δ-aminolevulinic acid into heme *in vitro* was decreased in vitamin B_6, niacin, pantothenic acid, riboflavin, and thiamine deficiencies, and this became more pronounced with the duration of the deficiencies. Glycine uptake was decreased more than δ-aminolevulinic acid uptake, and this was attributed primarily to low reticulocyte levels found in all the deficiencies.

These effects made it difficult to determine whether or not the more efficient incorporation of δ-aminolevulinic acid could in part be attributed to a specific biochemical lesion involving the vitamin as an enzymatic cofactor in the synthesis of δ-aminolevulinic acid from glycine and succinate. That this was so in vitamin B_6 deficiency was shown by the fact that the addition of pyridoxal phosphate stimulated the incorporation of glycine, but not of δ-aminolevulinic acid *in vitro*. It had no such effect in control blood.

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Vitamin B_{12} Absorption in Pyridoxine-Deficient Rats

Further Studies

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IN PREVIOUS REPORTS[1] from this laboratory it was demonstrated that pyridoxine deficiency results in an impairment of absorption of orally administered vitamin B_{12}. This phenomenon was demonstrated by urinary excretion and fecal excretion tests as well as by the measurement of radioactivity retained in certain organs of pyridoxine-deficient and pyridoxine-treated rats, orally fed with radioactive vitamin B_{12}. The impairment could be fully corrected by subsequent treatment with pyridoxine.

The manner in which the impairment occurs is not fully understood. The present report deals with a study to determine whether the impairment is due to the lack of secretion of adequate amounts of gastric juice—a source of intrinsic factor—or to the atrophy of some endocrine gland.

EXPERIMENTAL PROCEDURE

Preparation of Animals

Rats with Pyridoxine Deficiency. Young (six weeks old) as well as adult male rats (three months old) of the McCollum strain were used for this investigation. Animals were placed in individual screen bottom cages and kept on a casein-sucrose basal diet[2] with all known vitamins except pyridoxine. Half of the animals were given intraperitoneal injections three times a week of an aqueous solution of pyridoxine hydrochloride (1 mg in 1 ml). All animals were kept on the deficient diet until the untreated animals developed outward signs of deficiency, such as acrodynia, retardation of growth, and/or alteration in serum glutamic oxalacetic transaminase (SGOT) activity. Some biochemical data on our deficient and treated animals were also accumulated.

After Adrenalectomy. Two-month-old male rats were bilaterally adrenalectomized. They were allowed to recuperate from the operation and to rest for at least ten days before they were used. During this period, the animals were offered our stock diet and water containing 1 per cent sodium chloride. Intact male rats of the same age were used as controls.

After Hypophysectomy. Hypophysectomized male rats, purchased from the Charles River Breeding Laboratory, weighed 150 g at the time of experiment. According to the vendor these animals were 2–3 months old.

Materials

Adrenocorticotrophic Hormone (ACTH) and *Thyroid-stimulating Hormone* (TSH). The ACTH and TSH preparations were kindly supplied to us by Armour Research Laboratories.

Sources of Intrinsic Factor. Gastric juice from normal rats was obtained after histamine stimulation. Stomachs removed from stock animals were homogenized with three times its weight of a cold saline solution. These two preparations served as our sources of intrinsic

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factor. Only freshly prepared specimens were used for our study.

Chemical Procedures

The procedures used for the determination of SGOT activity[3] or vitamin B_{12} levels in sera[4] or vitamin B_{12} levels in liver[5] as well as for the estimation of vitamin B_{12}-binding capacity[6] have been published elsewhere in detail and will not be repeated here.

Gastric Secretion. The secretion of gastric juice of pyridoxine-deficient and treated animals in the fasting state was measured after ligating the pylorus under ether anesthesia. Gastric juice was collected with a syringe on the following morning, i.e. approximately 17 to 18 hours after ligation of the stomach. The total volume of gastric juice was estimated. Its pH and total vitamin B_{12}-binding power were measured.

Absorption of Vitamin B_{12}. The test animals were placed in individual metabolism cages. Feed and water were provided ad libitum. Each rat was force-fed with stomach tube 50 mμg of radioactive vitamin B_{12}-labeled with Co^{60} of specific activity of 1 μc/μg in 2 ml distilled water, with or without intrinsic factor preparation. Feces were collected in graduated bottles for 6 or 8 days, at the end of which time the animals were sacrificed under light anesthesia. The livers, kidneys, and gastrointestinal tracts of the rats were removed, cleaned with a normal saline solution, and finally solubilized by adding an aqueous solution of 30 per cent potassium hydroxide. All samples were then diluted to 50 ml for the measurement of radioactivity with a gamma scintillation counter.

RESULTS

In order to be certain that our animals are deficient in pyridoxine, body-weight changes and certain biochemical measurements of both pyridoxine-deficient and treated animals were compared. Results are given in Table I. Aside from the reported data on the decrease in the serum transaminase content, pyridoxine-deficiency resulted in low vitamin B_{12} levels in serum, but not in liver.

The analytical results on the composition of gastric juice of pyridoxine-deficient and treated animals are tabulated in Table II. These data demonstrate clearly that pyridoxine deficiency results in slight decrease in the volume of gastric secretion and increase in pH of the gastric juice, but a marked decrease in the total vitamin B_{12}-binding power.

Intrinsic factor aids absorption of vitamin B_{12} by man[7,8] and by rats.[9,10] Our data show that pyridoxine-deficient animals secrete slightly less gastric juice and markedly less vitamin B_{12}-binding substance, which is believed to be an essential property of intrinsic factor. Therefore it was of interest to determine whether the coadministration of vitamin B_{12} with intrinsic factor either from gastric juice or stomach homogenate of rats will improve the absorption of vitamin B_{12} by pyri-

TABLE I

Comparison of Pyridoxine-deficient and Pyridoxine-treated Rats

Treatment	Weight increase (g)	Plasma transaminase (units)	Vitamin B_{12} levels	
			Liver (mμg/g)	Plasma ($\mu\mu$g/ml)
Deficient	48 (6)	22.8 ± 4.5 (6)	161.0 ± 30.6 (9)	132.4 ± 12.8 (11)
Treated	124 (6)	87.0 ± 4.1 (6)	163.9 ± 24.6 (9)	194.7 ± 10.9 (11)

Eleven rats in each group were used at the beginning of the study. The number of specimens used for each measurement is indicated by the numbers in parentheses.

TABLE II

Some Properties of the Gastric Juice in Pyridoxine-deficient and Treated Rats

Treatment	Volume (ml)	pH	Total vitamin B_{12} binding power (mμg)
Deficient	4.7 (1.8–7.8)	1.65 (1.42–2.18)	10.7 (4.9–17.0)
Treated	6.5 (4.0–8.4)	1.49 (1.32–1.86)	34.4 (12.0–52.7)

Gastric juice was obtained 16 hours after ligation of pylorus. Vitamin B_{12} binding power was kindly assayed by Dr. Robert Davis, Veterans Administration Hospital, Baltimore, Md.

Four rats were used in each group for this study.

TABLE III

Absorption of Radioactive Vitamin B_{12}

Eight days' fecal excretion

Treatment	Radioactivity recovered as unabsorbed vitamin B_{12} (% of oral dose)
Deficient	74.9 ± 10.03 (4)
Treated	42.4 ± 2.00 (3)

Numbers in parentheses denote number of rats used.

doxine-deficient rats. The typical results of several such studies are summarized in Tables III, IV, and V. Table III demonstrates the impairment of the absorption of vitamin B_{12} due to pyridoxine deficiency. The pyridoxine-deficient animals absorbed on the average only 25 per cent of a 50 mμg dose of vitamin B_{12}, while the treated animals absorbed as much as 58 per cent. Although the magnitude of the difference in the absorption by these two groups of animals may vary somewhat from one experiment to another, consistent differences were observed in a series of many experiments.

In another study, pyridoxine-deficient and treated animals were divided each into three subgroups to receive 0, 1, and 3 ml of gastric juice of normal rats, respectively. The gastric juice was premixed with 50 mμg radioactive vitamin B_{12} and was then fed orally to the rats. Fecal excreta were collected for eight days, after which the animals were sacrificed and their livers, kidneys, and stomachs were removed. The radioactivity in all these specimens was measured. The results are tabulated in Table IV. There was no observable difference in the radioactivity in fecal matter or in the organs of the treated or deficient animals. Thus, feeding of intrinsic factor failed to improve the absorption of vitamin B_{12} by pyridoxine-deficient animals. Since the average body weight of pyridoxine-deficient animals was approximately one-half that of the treated animals, it is conceivable that a different conclusion might be drawn if our results were expressed in terms of millimicrograms of vitamin B_{12} per unit weight of organs. Nevertheless, such calculation likewise showed that the administration of gastric juice in quantities mentioned above resulted in no benefit to the pyridoxine-deficient or treated animals.

Beside gastric juice, stomach homogenate equivalent to one-half[9] of the rat stomach was administered together with vitamin B_{12} to pyridoxine-deficient and treated animals. The administration of stomach homogenate resulted in no difference in absorption of vitamin B_{12}.

Tauber[11] and associates demonstrated that the administration of histamine increases the absorption of vitamin B_{12} by the young subjects but not the old subjects. Since the latter group likewise show marginal pyridoxine-deficiency, it was, therefore, of interest to determine whether the administration of histamine to pyridoxine-deficient animals would increase the absorption of vitamin B_{12}. To this end, histamine or saline was injected to pyridoxine-deficient animals one hour prior to the oral feeding of radioactive vitamin B_{12}. To be certain that histamine effect lasted during the period when absorption of vitamin B_{12} remained critical, another dose of histamine was given four hours afterwards. Our results showed that the histamine-injected group absorbed 49

TABLE IV

Effect of Gastric Juice on Absorption of Radioactive Vitamin B_{12} and Residual Radioactivity in Organs[a]

Eight days' fecal excretion

Treatment	No. rats	Gastric juice (*ml*)	Radioactivity as vitamin B_{12}			
			Feces	Liver	Kidneys	Stomach
Deficient	4	0	56.8 ± 3.87	6.1 ± 0.71	5.3 ± 0.54	1.2 ± 0.33
	4	1	59.2 ± 2.84	7.1 ± 0.64	4.9 ± 0.57	1.4 ± 0.11
	4	3	52.9 ± 3.74	7.8 ± 0.71	5.9 ± 0.52	0.9 ± 0.16
Treated	3	0	52.7 ± 2.96	7.9 ± 0.42	5.3 ± 0.03	1.6 ± 0.05
	4	1	46.2 ± 2.57	8.7 ± 0.34	6.0 ± 0.35	1.1 ± 0.08
	4	3	42.9 ± 2.86	10.1 ± 0.64	7.1 ± 0.25	1.2 ± 0.06

[a] Percentage of oral dose.

per cent, and the saline injected group absorbed 45 per cent of the orally administered dose (50 mμg). Similarly, there was no difference in the radioactivity deposited in the target organs of the two groups of animals. Thus, histamine stimulation, under our experimental conditions, failed to enhance the absorption of vitamin B_{12} by pyridoxine-deficient rats.

The data presented above demonstrate that the prime factor in the impairment of absorption of vitamin B_{12} by the pyridoxine-deficient animals is not related to gastric juice secretion nor to any "deficiency" of intrinsic factor-like material. It remains, therefore, to determine whether pyridoxine deficiency may result in some endocrine disturbances which in turn affect vitamin B_{12} absorption. Consequently, the role of hypophyseal hormones on the absorption of vitamin B_{12} was examined. It was reported previously[12] that thyroid atrophy or removal can bring about a reduction in the absorption of vitamin B_{12}. However, there is no definite evidence that pyridoxine deficiency is accompanied by any atrophy of the thyroid. Efforts were made therefore, to determine whether other endocrine glands might cause the impairment of this absorption. Since adrenal insufficiency has been observed in connection with pyridoxine deficiency,[13,14] we investigated whether the adrenal may be involved in the absorption of vitamin B_{12} by pyridoxine-deficient animals. To this end, the absorption of vitamin B_{12} by adrenalectomized and stock animals was measured. Castrated and splenectomized rats were also included in this study in order to evaluate the relative specificity of adrenalectomy. The results tabulated in Table V demonstrate that the adrenalectomized rats absorbed less of the orally administered vitamin B_{12} than did the stock animals or the castrated rats. Splenectomy also reduced the absorption of vitamin B_{12}.

TABLE V

Effect of Adrenalectomy on Absorption of Radioactive Vitamin B_{12} by Stock Animals[a]

Treatment	Radioactivity as vitamin B_{12}		
	Feces	Liver	Kidneys
Adrenalectomy (6)	78.2 ± 2.04	3.7 ± 0.31	6.1 ± 0.50
Splenectomy (4)	75.0 ± 4.77	4.2 ± 0.37	2.9 ± 0.22
Castration (5)	41.1 ± 4.68	4.1 ± 0.58	5.5 ± 1.07
Control (5)	50.0 ± 4.78	3.6 ± 0.68	8.1 ± 1.38

[a] Percentage of oral dose.
Numbers in parentheses denote number of rats used.

TABLE VI

Effect of Adrenalectomy on the Absorption of Radioactive Vitamin B_{12} by Pyridoxine-deficient Animals[a]

Treatment	Radioactivity as vitamin B_{12}			
	Feces and gastrointestinal tract	Liver	Kidneys	Spleen
Adrenalectomy (4)	78.5 ± 4.49	8.4 ± 0.23	3.0 ± 0.56	0.38 ± 0.09
Control (4)	65.0 ± 4.05	10.9 ± 1.43	7.8 ± 0.56	0.50 ± 0.03

[a] Percentage of oral dose.
Numbers in parentheses denote number of rats used

The results given in Table VI demonstrate that adrenalectomy of pyridoxine-deficient animals reduces to a small extent the absorption of vitamin B_{12} as indicated by the fecal excretion and by the retention of radioactivity in various organs such as liver, kidneys, and spleen.

If the adrenal function of the rat can affect the absorption of vitamin B_{12}, it may be expected that hypophysectomy would also impair the absorption of vitamin B_{12}. To this end, hypophysectomized rats were divided into three subgroups and given treatments subcutaneously, as follows for ten days: group A, a normal saline solution; group B, 2 U.S.P. units of ACTH; and group C, 0.25 U.S.P. units of TSH. Our stock animals, group D, served as control. Approximately two weeks after treatment with hormone preparations, the vitamin B_{12} absorption test was conducted. The results tabulated in Table VII demonstrate that hypophysectomy results in poor absorption which can be improved by the injection of ACTH and TSH. Since commercial preparations of ACTH may be contaminated with TSH

TABLE VII

Effect of Hypophysectomy on Absorption of Radioactive Vitamin B_{12}[a]

Group	Treatment	Radioactivity as vitamin B_{12}		
		Feces	Liver	Kidneys
A	Hypox[b]-saline (6)	80.1 ± 7.0	2.2 ± 0.36	2.2 ± 0.42
B	Hypox-ACTH (7)	54.3 ± 7.3	7.8 ± 2.62	7.5 ± 1.63
C	Hypox-TSH (5)	50.5 ± 13.5	7.6 ± 2.82	6.2 ± 1.96
D	Stock diet (8)	60.7 ± 1.4	4.8 ± 0.82	4.2 ± 0.58

[a] Percentage of oral dose.
[b] Hypox = hypophysectomy.
Numbers in parentheses denote number of rats used.

or other pituitary hormones, or vice versa, an examination of the weights of the thyroids and adrenals of the animals in the four groups indicated, however, that the specimens were relatively free from contamination (Table VIII). It can then be concluded that ACTH and TSH both play roles in controlling the absorption of vitamin B_{12}.

Since atrophy of the adrenal cortex may decrease the secretion of many hormones by the adrenals, it was of interest to determine whether the impairment of vitamin B_{12} absorption is due to the lack of cortisone. This hormone was injected into pyridoxine-deficient and stock animals. The absorption was measured by the fecal excretion test and by the measurement of radioactivity in different organs following the oral administration of radioactive

TABLE VIII

Weights of Thyroid and Adrenal Glands of Hypophysectomized Rats under Various Treatments

Group	Treatment	Thyroid		Adrenals	
		Average (mg)	Range (mg)	Average (mg)	Range (mg)
A	Hypox[a]-saline (6)	4.8	3–6	8.4	7–10
B	Hypox-ACTH (7)	5.1	3–7	15.6	13–19
C	Hypox-TSH (5)	8.6	5–11	4.5	3–6
D	Stock diet (8)	12.3	9–14	29.8	22–50

[a] Hypox = hypophysectomy.
Numbers in parentheses denote number of rats used in each group.

TABLE IX

Effect of Cortisone on Vitamin B_{12} Absorption[a] in Pyridoxine-Deficient Rats[b]

Treatment	Radioactivity as vitamin B_{12}		
	Feces	Liver	Kidneys
Cortisone (3)	44.8 ± 3.80	7.2 ± 0.58	18.8 ± 1.40
Saline (3)	60.8 ± 2.10	5.6 ± 0.75	15.7 ± 3 20

[a] Percentage of oral dose.
[b] Vitamin B_{12} supplied in the ration.
Numbers in parentheses denote number of rats used.

vitamin B_{12}. The results tabulated in Table IX demonstrate that the injection of cortisone improves the absorption of vitamin B_{12}. These data taken as a whole, suggest a definite role of adrenal glands on absorption of vitamin B_{12}, mediated possibly by 11-oxycorticosteroids.

DISCUSSION

The importance of the status of the adrenal gland in the absorption of vitamin B_{12} is demonstrated by the diminution of absorption after adrenalectomy and by the improvement of absorption upon the injection of cortisone. The latter compound affects not only pyridoxine-deficient animals but also our stock animals. The impairment of the absorption of vitamin B_{12} after hypophysectomy can be corrected by the administration of ACTH as well as by the administration of TSH. However, the injection of ACTH alone apparently brings the vitamin B_{12} absorption to normalcy. If thyrotropic hormone, which is likewise essential for the absorption of vitamin B_{12}, plays a role independent of that of ACTH, it should not be expected that the administration of ACTH alone should correct the absorption almost completely, unless our ACTH preparation might be contaminated with thyrotropic hormone in an amount sufficient to improve the absorption of vitamin B_{12} but inadequate to stimulate hypertrophy of thyroid of the hypophysectomized rats.

Involvement of adrenals in pyridoxine deficiency was suggested by electrolyte imbalance[14,17] increase in adrenal size and cholesterol content in pair-fed rats,[14] and histochemical changes.[13] These alterations suggest some impairment of adrenal cortical function in pyri-

doxine-deficient animals. Furthermore, most of the changes can be readily corrected by administration of cortisone but not by desoxycorticosterone acetate. Our findings at least in part suggest this impairment of vitamin B_{12} absorption can be the result of the adrenal insufficiency.

According to a report of Strauss and Brokaw,[18] adrenocortical function in patients with addisonian anemia is impaired. It is interesting to note that Addison originally described adrenal insufficiency and pernicious anemia as closely related conditions.[19] Prednisone was recently reported to enhance vitamin B_{12} absorption in one patient with addisonian anemia.[20] Cortisone can also enhance vitamin B_{12} absorption in tropical sprue.[21,22] Whether this enhancement of absorption of vitamin B_{12} following cortisone administration is only specific for vitamin B_{12} or other nutrients as well has not been explored.

The influence of adrenal cortical hormones on gastric secretion has received considerable attention. Adrenalectomy produces an increase in pH and mucin content of fasting gastric juice, but a reduction in the pepsin and renin activities, even under the stimulus of histamine and cholinergic drugs. Administration of cortisone or ACTH brings about an increase of pepsin and basal and nocturnal secretion, with augmentation in acidity, pepsin activity, and total volume of gastric juice both in normal subjects and in patients with duodenal ulcer.[23,24] Our pyridoxine-deficient rats may well be suffering from certain degrees of adrenal dysfunction followed by secondary changes in gastrointestinal tract. The latter effect may play a more direct role in the impairment of absorption of vitamin B_{12}.

SUMMARY

Pyridoxine deficiency in rats results in an impairment of absorption of orally administered vitamin B_{12}. The manner in which the impairment occurs is not fully understood. In this present study, data are presented to determine whether the impairment might be due to the insufficiency of secretion of gastric juice or the atrophy of some endocrine glands. It was found that pyridoxine-deficiency resulted in a lesser secretion of gastric juice and a marked reduction of vitamin B_{12}-binding power. However, the coadministration of vitamin B_{12} with intrinsic factor, either from stomach homogenate or from rat gastric juice, failed to improve the absorption of orally administered vitamin B_{12}.

Studies were conducted to examine the possibility of endocrine dysfunction as a cause of the impairment of vitamin B_{12} absorption. It was found that in addition to the thyrotrophic hormone the adrenal cortical hormones can affect the absorption of vitamin B_{12}. Thus, hypophysectomy or adrenalectomy impairs the absorption of orally administered vitamin B_{12}. This absorption can be improved by the administration of cortisone to pyridoxine-deficient and stock animals or by injection of ACTH or TSH to hypophysectomized animals. These data taken as a whole indicate that pyridoxine deficiency results in adrenal atrophy, which in turn brings about a decrease in the absorption of vitamin B_{12}.

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Studies on the Role of Intrinsic Factor in Vitamin B_{12} Absorption, Transport, and Storage

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A LARGE BODY of research has led to general acceptance of the fact that intrinsic factor is responsible for effecting the transfer of physiologic amounts of vitamin B_{12} across the intestinal mucosa and into the blood stream.[1] However, despite intensive research in many laboratories, it has not yet been possible to demonstrate the mechanism of action of intrinsic factor or, indeed, to prepare intrinsic factor in pure form.[2]

The possible existence of an extragastric intrinsic factor was suggested in a report by Callender and Lajtha,[3] indicating that maturation of megaloblasts *in vitro* could be brought about by normal gastric juice (intrinsic factor) and vitamin B_{12}, but not by either agent alone. As indicated in a review[4] of this subject, some workers, but not all, have been able to confirm this finding, and it is not generally accepted at present.

Early in 1957, Miller, Raney and Hunter[5] reported that the vitamin B_{12}-Co^{60} uptake of rat liver slices could be enhanced by 50 per cent if hog intrinsic factor concentrate (HIFC) were added to the incubation medium. We were at that time engaged in studies of the vitamin B_{12}-Co^{60} uptake of rat liver slices under various experimental conditions, and it was possible to confirm this finding within 24 hours of the time the abstract was brought to our attention by W. L. Williams. Indeed, by using a physiologic level of vitamin B_{12} and by thoroughly washing the slices after incubation, it was possible to demonstrate a tenfold enhancement of vitamin B_{12} uptake in the presence of HIFC.[6,7]

One fact was outstanding. In Krebs-Ringer-Phosphate solution, the conventional incubation medium for our work with tissue slices, the effect of HIFC was much less than in Hastings Bicarbonate-Pyruvate medium, used by Miller and his associates. This point is illustrated in Table I. Obviously, there was some part of the medium involved in this difference, and by eliminating each component of the Hastings medium separately in a series of experiments, it was possible to demonstrate that calcium was the

TABLE I

Vitamin B_{12} Uptake by Rat Liver Slices

In Krebs-Ringer Phosphate:	
Without HIFC added	216 ± 6
With HIFC added	743 ± 17
In Hastings Bicarbonate Pyruvate:	
Without HIFC added	138 ± 9
With HIFC added	1324 ± 35

HIFC potent in daily dose, 0.5 mg
HIFC: vitamin B_{12} ratio 310:1 (by weight)

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TABLE II

Vitamin B_{12} Uptake by Rat Liver Slices

In Hastings Bicarbonate:	
Without HIFC added	387, 349
With HIFC added	2634, 2599
In Hastings Bicarbonate without calcium:	
Without HIFC added	340, 404
With HIFC added	40, 35

HIFC potent in daily dose, 0.5 mg
HIFC: vitamin B_{12} ratio 310:1 (by weight)

vital factor. (The calcium concentration of the Hastings medium is considerably higher than that of the Krebs-Ringer-Phosphate solution.) This is illustrated in Table II.

Note that in the absence of calcium, not only is vitamin B_{12} uptake by rat liver slices not enhanced in the presence of HIFC; but it is also actually reduced. Subsequent experiments have indicated that the mechanism of this reduction is that the vitamin B_{12} was all bound to the HIFC, but in the absence of calcium the combined product could not attach to the liver slice. Thus there was not only no intrinsic factor effect, but also no diffusion of free vitamin B_{12} into the slices, as occurred with control slices.

A study of time and temperature factors was conducted. As shown by Miller *et al.*[5,8] the enhanced vitamin B_{12} uptake produced by HIFC was a function of time. More strikingly, however, it did not appear to be a function of temperature.[6,7] As Figure 1 shows, even after three hours' incubation, the HIFC effect is almost identical at 3°C to that observed at 37°C. Reduction of vitamin B_{12} uptake at the lower temperature occurred in the control slices rather than those incubated with intrinsic factor concentrate.

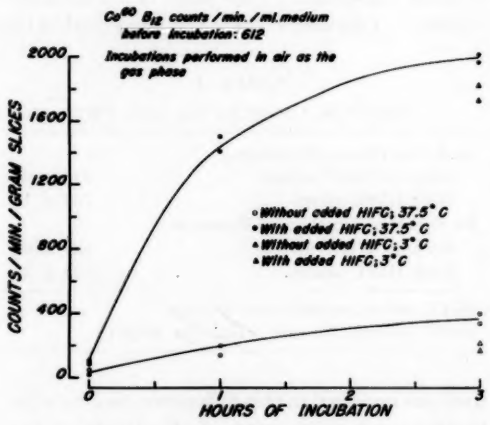


Fig. 1. Effect of time and temperature of incubation on uptake. (From HERBERT, V.: *J. Clin. Invest.* 37:646, 1958.

Since the HIFC effect was not dependent upon temperature, and was not diminished by metabolic inhibitors such as 2,4-dinitrophenol,[6,7] it seemed likely that we were dealing with a physical phenomenon on the surface of the cell. If the intrinsic factor-vitamin B_{12} complex were held to the cell wall by calcium, it seemed reasonable to believe that a calcium-chelating agent such as ethylenediaminetetraacetate (EDTA) might release the complex.

Liver slices were incubated with vitamin B_{12}-Co^{60} and HIFC. They were then washed and aliquots incubated with EDTA added to the incubation medium. As suspected, marked removal of $cobalt^{60}$ from the slices occurred.[6,7] This of course did not prove that the vitamin B_{12}-Co^{60} was complexed to intrinsic factor on the cell wall; nor indeed that the EDTA was not acting on a bond within the cell rather than on the cell surface. It was suggestive enough to warrant further experimentation along similar lines of reasoning.

The effect of various concentrations of HIFC on vitamin B_{12} uptake by liver slices was now studied. It was found that for each different preparation of HIFC there was a single specific concentration producing maximal vitamin B_{12} uptake (Fig. 2).[9,10] Either increasing or decreasing the concentration of HIFC from this maximal point resulted in a lesser enhancement of vitamin B_{12} uptake. The more potent the HIFC in human subjects with pernicious anemia as a source of intrinsic factor, the lower the concentration of that HIFC producing maximal enhancement. (In Figure 2, HIFC #A is more potent than HIFC#B.)

This finding suggested that there might be receptors for intrinsic factor on rat liver slices. If there were such receptors in specific number, and each receptor could accommodate one intrinsic factor molecule (either free or with vitamin B_{12} attached to it), then there would be a

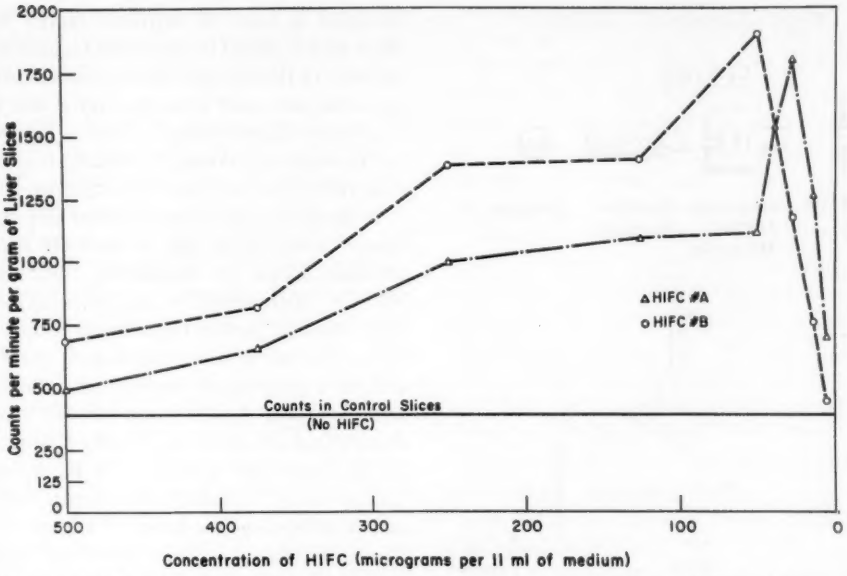


Fig. 2. Co^{60}-B_{12} uptake by rat liver slices incubated concurrently in Co^{60}-B_{12} and HIFC (in decreasing concentrations).

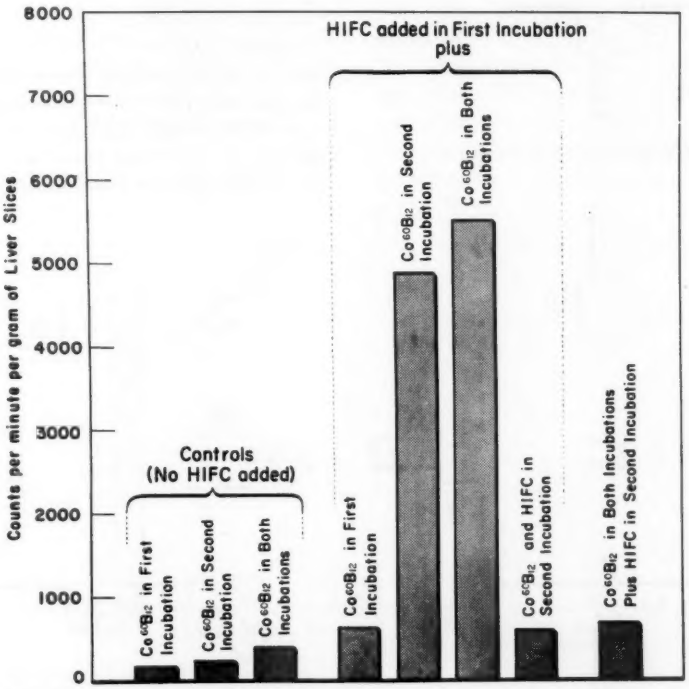
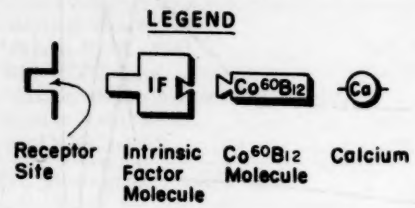


Fig. 3. Simultaneous vs. sequential incubation of rat liver slices in Co^{60}-B_{12} and HIFC.

Key to Figures 4, 5, and 6.



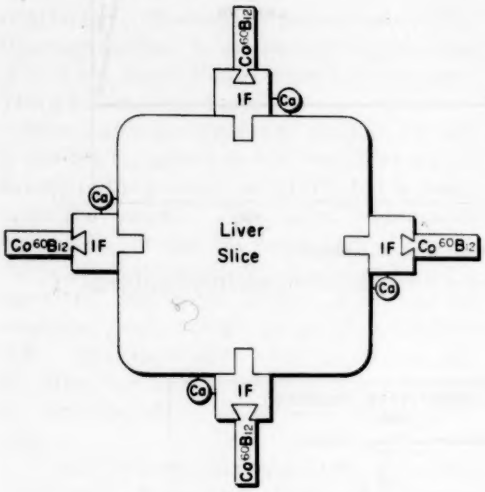


Fig. 4. Optimal HIFC concentration.

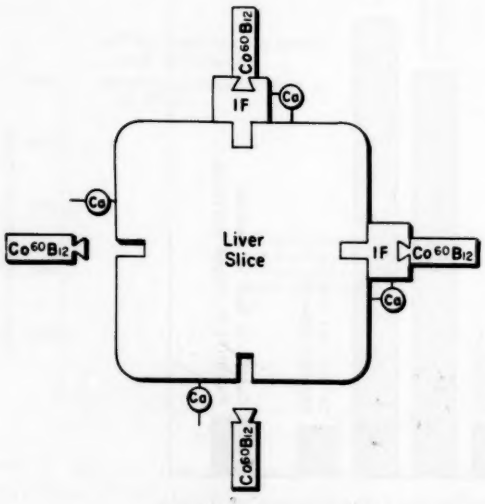


Fig. 5. Half optimal HIFC concentration.

maximal uptake of intrinsic factor by liver slices which would be the same regardless of the potency of the intrinsic factor concentrate used, provided sufficient intrinsic factor was present to saturate the receptors. (Note in Figure 2 that the maximum vitamin B_{12} uptake is similar for two HIFC preparations of different potency.)

If receptors for intrinsic factor did exist, one would expect to be able to saturate them with intrinsic factor by incubating liver slices in HIFC. Such receptors should be expected to hold intrinsic factor tenaciously despite washing of the slices after incubation with HIFC, and in a subsequent incubation of such slices with vitamin B_{12}-Co^{60} one should observe a marked enhancement of vitamin B_{12} uptake due to binding of the vitamin to intrinsic factor on the receptors. Exactly such results were noted in actual studies, as shown in Figure 3. Note especially that when liver slices were incubated first in HIFC and subsequently in vitamin B_{12}-Co^{60}, their uptake was multifold that of slices incubated in both agents together, which in turn was multifold that of slices incubated in vitamin B_{12} alone.

Figures 4, 5, and 6 are a pictorial representation of the hypothesis which seems to explain the various phenomena presented. Consider that the liver slice has four receptors for intrinsic factor, and that there are four molecules of vitamin B_{12}-Co^{60} in the surrounding medium.

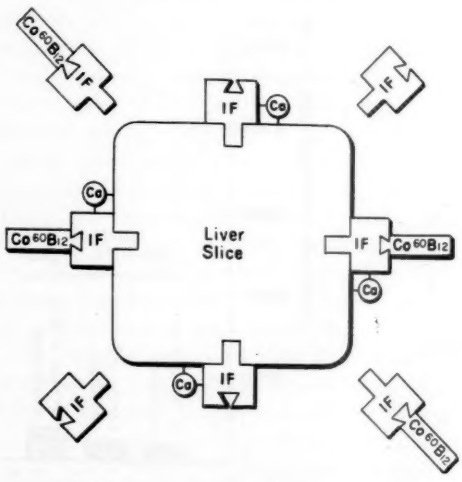


Fig. 6. Twice optimal HIFC concentration.

If four molecules of intrinsic factor are now introduced into the medium, the result will be as depicted in Figure 4—maximal enhancement of vitamin B_{12} uptake.

Should only half the optimal amount of intrinsic factor be present, half-optimal uptake of vitamin B_{12} will occur, as depicted in Figure 5.

Now, the situation in which a twice-optimal concentration of intrinsic factor is present, we observe (Fig. 6) the uptake by the slices of *only a half-optimal amount of vitamin B_{12}*. Comprehension of this latter concept is of particular importance, for it may explain many phenomena relating to intrinsic factor. This subject will be explored more fully shortly.

The question of the location of the receptors was approached by cell fractionation. Ellenbogen and we,[11,20] and Minard and Wagner[12] have found that the enhancing effect of intrinsic factor concentrates on vitamin B_{12} uptake is demonstrable in the precipitate of a liver homogenate after centrifugation at low speeds. To the contrary, Miller and Hunter[13] find that "intrinsic factor containing materials will stimulate receptor proteins to combine vitamin B_{12} of tissues as well as serum. Further (they report), the receptor proteins of tissues are located intracellularly in the microsome and soluble protein fraction of liver homogenates."

In our studies of fractions of liver homogenates, we find the intrinsic factor dependent-calcium dependent-EDTA reversible vitamin B_{12} uptake to occur in the cell debris (cell walls and nuclei fraction), and not in either the supernatant soluble protein fraction or the microsomes. Indeed, we find that the excess vitamin B_{12} bound in the supernatant fraction in the presence of intrinsic factor concentrates is explainable on the basis of direct binding to such concentrates. The study suggesting otherwise[13] lacked an important control: determination of the amount of vitamin B_{12}-Co^{60} precipitated on $(NH_4)_2SO_4$ fractionation from a solution containing only intrinsic factor. We have performed experiments with such controls and find that the "intracellular receptor proteins" appear superfluous. This type of experiment appears to do no more than confirm the fact that intrinsic factor precipitates in $(NH_4)_2SO_4$ of 35 to 55 per cent concentration.[2] There appears to be no need to invoke any other explanation such as intrinsic factor "stimulating the intracellular receptor proteins to combine with vitamin B_{12}."[13] Present knowledge is such as to support the hypothesis that there are receptors for intrinsic factor, located probably on the cell wall of the liver, and that any uptake of vitamin B_{12} by other subcellular fractions is due to direct binding unrelated to intrinsic factor. This, of course, does not preclude the possibility that intracellular transport, storage, and utilization of vita-

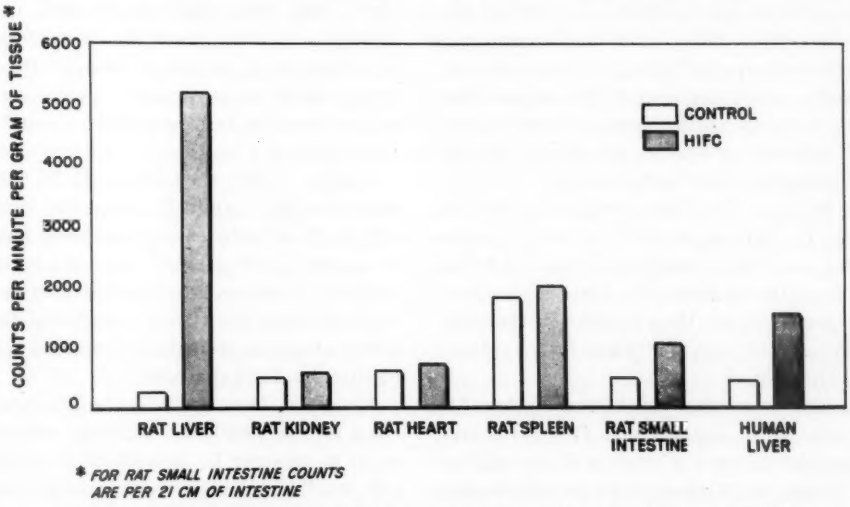


Fig. 7. *In vitro* organ uptake of Co^{60}-B_{12}, without and with preincubation in HIFC.

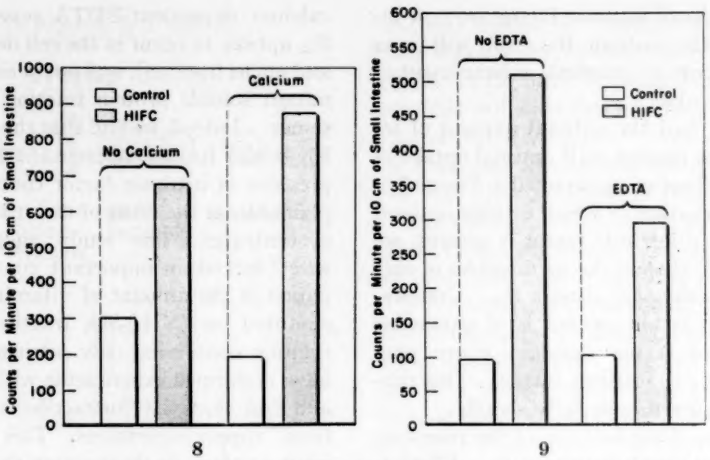


Fig. 8. Rat small intestine Co^{60}-B_{12} uptake, without and with preincubation in HIFC, in the absence and in the presence of added calcium.

Fig. 9. Rat small intestine Co^{60}-B_{12} uptake, without and with preincubation in HIFC, with final incubation without and with EDTA.

min B_{12} *in vivo* may require the presence of intrinsic factor or an intrinsic factor-like material.

Despite the fact that the precipitate of a liver homogenate may prove to be a simpler tool in the assay of intrinsic factor preparations (as experiments so far indicate it is), we still use liver slices in most of our studies. One reason for this is that we are interested in both the intrinsic factor-dependent and the intrinsic factor-independent mechanisms of vitamin B_{12} uptake by cells.[14] The latter mechanism cannot be completely studied in a system of nonintact cells; all our evidence to the present time suggests that this mechanism is diffusion into the cell, followed by binding of vitamin B_{12} by various components of the cell content.

Now, we had a hypothesis suggesting the existence on rat liver slices of receptors for intrinsic factor, and these receptors appeared to be specific for intrinsic factor.[10] Did they exist in other organs? Were they present in the only generally accepted site of intrinsic factor action, the small intestine?

Using the sequential incubation system,[9,10] it was possible to demonstrate (Fig. 7) a tremendous enhancement of vitamin B_{12} uptake by rat *or human* liver slices after preincubation with HIFC. Only a slight, and not clearly significant, enhancement of vitamin B_{12} uptake by slices of rat kidney, heart, or spleen was noted.[15,16] In addition Figure 7 shows that the vitamin B_{12} uptake of the tissue slices *in the absence of preincubation with HIFC* is lowest in liver, greater in kidney, still more in heart, and by far greatest in spleen. The significance of this is not yet determined, but it is a subject for further study. One wonders, for example, if the marked control uptake in spleen slices may mean that an intrinsic factor-like substance could already be present on spleen receptors ready to take up vitamin B_{12} and convey it into blood formation. (A similar marked control uptake is observed in normal human bone marrow.)

Figure 7 also shows that HIFC markedly enhances the vitamin B_{12} uptake of segments of rat small intestine. This striking finding led to studies with everted sacs of rat small intestine,[17,18] with results so similar to those with rat liver slices that it was concluded the mechanism of action of intrinsic factor was probably identical in both systems.

Figure 8 shows that in the absence of calcium in the incubation medium, the enhancement of vitamin B_{12} uptake by everted sacs of rat small intestine after preincubation with HIFC is only about half that which occurs in

the presence of calcium. (It is quite possible that if the everted sacs could be washed completely free of calcium, all uptake due to preincubation with HIFC could be eliminated, as it can with liver slices.) Note that the control uptake of everted sacs is greater in the absence of calcium. This may be due to the anti-inflammatory action of calcium, in the absence of which agent cell permeability may be increased.

Figure 9 demonstrates the marked reversibility of the HIFC effect by subsequent incubation in EDTA-containing medium. Again, the EDTA effect, while clearly and reproducibly present, is not as striking as in experiments with liver slices. We believe that this may be partly a function of the fact that the everted sac of small intestine has a coating of mucus and does not offer the smooth surface and 0.5 mm thickness of the liver slice, from which exchange with the surrounding medium is much easier.

It was thought that if indeed the mechanism of intrinsic factor action in the human intestine was calcium-dependent, vitamin B_{12} deficiency should develop in human subjects taking an oral calcium-chelating agent to tie up calcium in the intestine. Sodium phytate is such an agent, and has been used over prolonged periods in some studies.[19]

When phytate was given in 1.5 g doses every four hours for ten doses, it was found that vitamin B_{12} given 15 minutes after the seventh dose was not absorbed as well as vitamin B_{12} given in the absence of "priming" with phytate. This was true both for normal subjects and for patients with pernicious anemia given oral vitamin B_{12}-intrinsic factor combinations.[20] In addition, a decrease in serum vitamin B_{12} levels was noted in patients on prolonged phytate therapy; this decrease was proportional to the length of time on phytate and developed as slowly as do low vitamin B_{12} levels in patients with total gastrectomy.[20,21]* Furthermore, the early hematologic and neurologic signs usually associated with developing vitamin B_{12} deficiency were noted in these latter patients.[21]

* Patients were studied through the courtesy of Dr. P. H. Henneman.

The ointment would be perfect except for one big fly—all five patients on prolonged phytate therapy had normal amounts of radioactivity in their urine after oral administration of vitamin B_{12}-Co^{58}. It has not yet been determined that the radioactivity in the urine is vitamin B_{12} and not some product of vitamin B_{12} breakdown brought about by phytate; nor has it been determined if prolonged administration of phytate may result in some substance in serum which could inhibit microbial growth and give a false low microbiologic assay for vitamin B_{12}. At present we can neither prove nor disprove intrinsic factor dependence on calcium in the human intestine, and we hope others will explore this avenue further. We do at present believe that such dependence exists.

It seemed logical that the effect of intrinsic factor on vitamin B_{12} uptake by rat liver slices had physiologic significance, and that such significance may easily take the form of a circulating intrinsic factor (or intrinsic factor-like material) involved in the selective deposition of vitamin B_{12} in the liver. However, Miller *et al.*[5] had reported that serum did not enhance vitamin B_{12} uptake by rat liver slices. We reasoned that this failure may have been due not to lack of such a substance in serum, but to the presence in serum of other vitamin B_{12}-binding materials. Using sequential incubation, it should be possible to have this intrinsic factor-like material bind to liver slice receptors, and then wash away all other vitamin B_{12}-binding materials. After the washing, the slices should demonstrate enhanced vitamin B_{12} uptake.

Figure 10 shows that this in fact was the case. Note that 1 ml of serum samples from patients without or with pernicious anemia all were able to slightly enhance vitamin B_{12} uptake by liver slices in the sequential incubation system, and a fifteenfold concentrate of normal serum markedly enhanced uptake (to a degree equal to that reported for intrinsic factor by Miller *et al.* in their original communication[5]). Subsequently it has been possible to demonstrate similar significant enhancement by a fifteenfold concentrate of pernicious anemia serum.[22] These findings

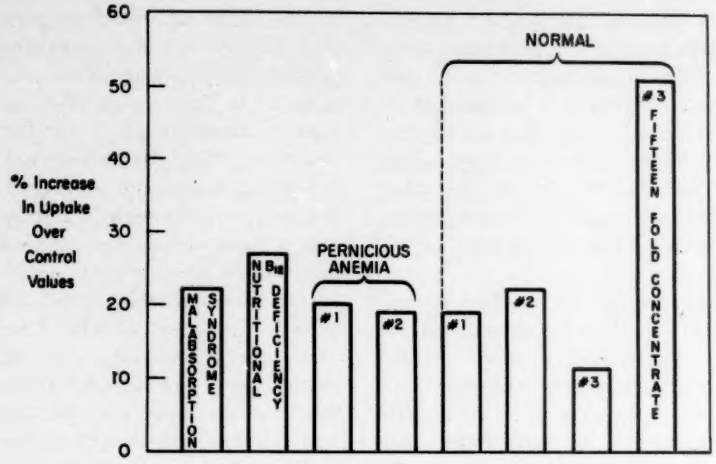


Fig. 10. Effect of human serum on Co^{60}-B_{12} uptake by rat liver slices.

lend credence to the possibility that there may indeed be a circulating intrinsic factor-like material, as speculated herein.

Let us now explore a subject touched on previously. This is the sequential incubation system as opposed to incubation of liver slices (or homogenates) in both intrinsic factor concentrate and vitamin B_{12} at the same time. Recall that our hypothesis, based on a fairly decent body of data, is that there are receptors for intrinsic factor on rat liver, and that these receptors are of a certain limited number. Remember too that intrinsic factor has not yet been prepared in pure form, and that all intrinsic factor concentrates so far made from either hog, human, or other species sources are contaminated to varying degrees. These contaminants may include various nonintrinsic factor substances capable of binding vitamin B_{12}, and possibly even substances capable of inhibiting intrinsic factor action by means other than competing for available vitamin B_{12}.

With these possibilities in mind, it is easy to explain why human gastric juice could not be assayed for intrinsic factor in a liver slice system by Johnson and Driscoll,[23] and could be so assayed by us.[9,10] They used simultaneous incubation; we used sequential. They were therefore plagued by contaminants of human gastric juice; we were not, since after incubating liver slices with the human gastric juice to bind intrinsic factor to the receptors, we washed the slices free of contaminants (including any excess intrinsic factor) prior to incubating them with vitamin B_{12}. Similar reasoning led to our using sequential incubation to demonstrate an intrinsic factor-like material in human serum, which was not demonstrable[5] in a system of incubation of both serum and vitamin B_{12} simultaneously. Using only simultaneous incubation, the conclusion was reached that "gastric juices from animal sources other than the hog have different effects on vitamin B_{12} uptake by liver slices and this is not correlated with intrinsic factor activity in the human."[23] When sequential incubation is used, however, one finds that various species of gastric juice enhance vitamin B_{12} uptake by rat liver slices. This includes rat, hog, and human gastric juices.

In short we believe at present that the sequential assay system is a measure *solely of the intrinsic factor content and intrinsic factor inhibitor of any gastric juice or intrinsic factor concentrate* and of the number of receptors for intrinsic factor. The simultaneous assay system, on the other hand, measures *the end result of the interplay of many factors:* amount of intrinsic factor in the concentrate, amount of nonintrinsic factor vitamin B_{12}-binding materials, amount of intrinsic factor

inhibitor (if such exists apart from nonintrinsic factor vitamin B_{12}-binders), and the number of receptors for intrinsic factor.

Both systems are useful. Since the *simultaneous* incubation system may measure the end result of the interplay of the various factors present in a given intrinsic factor concentrate, it may be of limited usefulness for *in vitro* determination of the *clinical potency* of hog intrinsic factor preparations (provided the amount of intrinsic factor in the preparation is not greater than the number of receptors, so that the intrinsic factor then "inhibits itself," as illustrated in Figure 6).

The *sequential* incubation system is probably preferable *for determining whether or not any given material contains intrinsic factor*, since this system ignores contaminants. (Here, too, excess intrinsic factor should be avoided. While small excesses will be washed off prior to incubation with vitamin B_{12}, we have suggestive evidence that an aliquot of large excesses may remain with the tissue during washing. This aliquot may then reduce the subsequent vitamin B_{12} uptake by the tissue as the excess intrinsic factor gradually goes into solution in the incubation medium, taking vitamin B_{12} with it.) The sequential incubation system is also probably of greater value in determining *purity* of a given intrinsic factor concentrate, and we are using it in attempts to isolate a purified intrinsic factor.

The usefulness of the sequential assay system for HIFC is illustrated in Figure 11. There is a close relative correlation of *in vitro* and *in vivo* potencies, but the absolute correlation is not as great. We believe this latter is due to different relative amounts of nonintrinsic factor vitamin B_{12}-binders in the various preparations, which would affect the amount of the vitamin absorbed from the human intestine, but not the amount taken up by the rat liver slice (in a sequential incubation system).

It should be mentioned that the amount of vitamin B_{12} uptake enhancement produced by HIFC per unit weight of rat liver homogenate is considerably greater than that per unit weight of rat liver slices. This is still in conformity with the hypothesis that the receptors

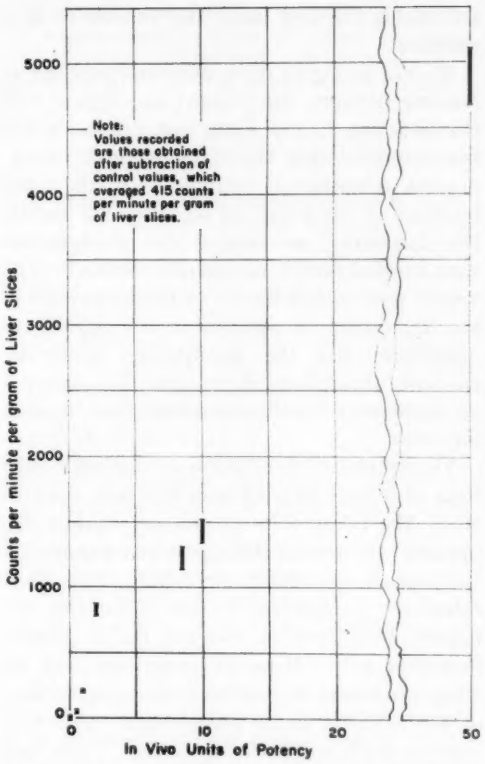


Fig. 11. Comparison of *in vitro* and *in vivo* assay of HIFC preparations.

are on the cell wall, since the cell surface area exposed to the incubation medium in a homogenate is greater than that of a slice.

A strong word of caution should perhaps be interposed here. We have spoken freely of measuring "intrinsic factor" in these studies. It must be emphasized that intrinsic factor has not yet been isolated in pure form, and until that day it must be kept in mind that we might be measuring nothing more than a contaminant common to all the intrinsic factor preparations so far produced, and also common to various gastric juices. While enhancement of liver slice-vitamin B_{12} uptake cannot be demonstrated with gastric juice from patients with pernicious anemia but is demonstrable with normal gastric juice,[10] this is not conclusive proof that we are indeed measuring intrinsic factor.

We should like to mention a few lines of study

now being pursued using the various *in vitro* systems.

We are trying to draw as many parallels as possible between the everted sac system and the situation in the living rat. Since it has been reported that the major intrinsic factor-dependent uptake of vitamin B_{12} in the small intestine of the living rat occurs in the middle two quarters,[24] we studied this phenomenon with everted sacs of rat small intestine.[25] The results were highly similar to those obtained in the living rat; in addition it was possible to determine that the *non*intrinsic factor-dependent absorption of vitamin B_{12} occurred approximately equally in each segment of small intestine.

The sequential incubation system with both liver slices and everted sacs has been used to study the effect of D-sorbitol on vitamin B_{12} uptake. Interest in this agent was aroused by the reports of Chow, Greenberg, and their respective co-workers[26,27] that D-sorbitol enhanced absorption of vitamin B_{12} in normal men and rats. However, D-sorbitol had no effect on either the intrinsic factor-dependent or the intrinsic factor-independent uptake of vitamin B_{12} in our *in vitro* systems. It also had no effect on vitamin B_{12} absorption by patients with pernicious anemia.[28] We feel justified at this time in accepting the possibility that the mode of action of D-sorbitol is solely to enhance the secretion of intrinsic factor.[29,30]

Another line of investigation is study of the site or sites on the vitamin B_{12} molecule involved in its binding to intrinsic factor in our *in vitro* systems.

Dr. Lester Smith has generously provided us with some of the anilide and the ethylamide of the monocarboxylic acid of vitamin B_{12}.[31] We found that in the liver slice system, intrinsic factor showed only a very slight selectivity for vitamin B_{12} over either of these agents.[22] We conclude that binding of vitamin B_{12} to intrinsic factor in the slice system does not involve only one of the labile amide groups and no other part of the molecule. Our results do, however, tend to support Smith's deduction[31] that "these substituted amides can compete successfully with the vitamin for binding sites on the specific protein."

Further studies with the substituted di- and tri-carboxylic acids of vitamin B_{12} will be pursued as soon as these agents (and other chemically altered vitamin B_{12} molecules) become available to us. We have the hearty cooperation of Dr. Herman Baker and his associates in this work; they are able to carry out parallel studies of each agent in terms of its effect on the growth of four different vitamin B_{12}-requiring microorganisms.

We hope in these studies, among other things, to find a potent anti-tumor agent. That "anti-B_{12}" which intrinsic factor cannot distinguish from vitamin B_{12}, and which will markedly inhibit the growth of the "humanoid" *Ochromonas Malhamensis*, will theoretically be such a potent agent. We have already initiated studies in patients with leukemia using the anilide and the ethylamide, which to a significant degree appear to have these properties. Hopefully, tumor tissue will rapidly take up the anti-vitamin, and then die because of failure of the anti-vitamin to perform the basic biochemical functions of vitamin B_{12}. Hopefully too, residual anti-vitamin could be rapidly washed out of the patient by the administration of large doses of vitamin B_{12}, at a point where major damage would have been done to rapidly growing cells and only slight damage to more slowly proliferating tissue.

Still another line of inquiry has already borne fruit. Since we believed the sequential incubation system with liver slices or homogenates measures the quantity of radioactive vitamin B_{12} taken up by a *specific number of molecules* of intrinsic factor on a specific number of receptors, it was logical to believe that we could rapidly assay the vitamin B_{12} content of biological fluids by determining the reduction in uptake of radioactivity when such fluids were added to the system. The nonradioactive free vitamin B_{12} in such fluids would compete in a 1:1 ratio with radioactive vitamin B_{12}. Such seems to be the case,[22] and we are at present further developing this procedure.

To conclude by referring back to the title of this presentation: What is the role of intrinsic factor in vitamin B_{12} absorption, transport, and storage? It is well documented that in-

trinsic factor makes it possible for physiologic amounts of vitamin B_{12} to be absorbed from the gastrointestinal tract. Evidence has been presented here that perhaps intrinsic factor may also be involved in the transport of vitamin B_{12} to the liver, and its selective deposition therein. Whether this is so remains to be conclusively determined, as does the possible existence of a circulating intrinsic factor.

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Serum Cholesterol Response in Man to Oral Ingestion of Arachidonic Acid

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IT IS NOW CLEAR that fats in the diet of man have important effects on the concentration of cholesterol and related lipids in the blood. and that the effects are related to the character as well as amount of the fatty acids in the diet. In general, when one fat is isocalorically substituted for another fat or for carbohydrate in the diet of man, the serum cholesterol response is the resultant of the fact that the common saturated fatty acids tend to raise the serum cholesterol, linoleic acid (or polyene in general?) tends to lower it, while oleic acid has substantially no effect.[1,2] The depressant effect of polyene or of linoleic acid upon serum cholesterol has been discussed frequently as being related to the "essentiality" of these fatty acids as demonstrated by other effects in experiments on animals receiving highly artificial diets extremely low in polyunsaturated fats.[3,4]

In view of the fact that arachidonic acid is widely believed to be the most active or effective of the fatty acids in regard to "essentiality," it is interesting to examine its effect, when provided in the diet, on the serum cholesterol level in man. The present paper is a report of a controlled experiment on men receiving a dietary supplement of arachidonic acid. By inference from the discussions in some of the recent literature, it might be expected that arachidonic acid would have some remarkable capacity to lower the serum cholesterol level.[5] The findings reported here do not correspond to such expectations.

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SUBJECTS, DIET AND PROGRAM

Nine men, aged 40 to 58 years, long domiciled at the Hastings State Hospital, were selected as subjects for this study on the basis of stability and cooperativeness demonstrated in previous studies, and the absence of signs of physiologic or biochemical abnormality. After preliminary study while the men subsisted on a standardized "house" diet, they were assigned to three groups of three men each, matched in age, relative body weight, and serum total cholesterol concentration on this standard diet. Thereafter for 70 days all men were maintained on a constant diet consisting of only two alternating daily menus. Each man was weighed, nude, before breakfast every day and, when necessary to maintain weight constancy, individual adjustments were made in the amounts of bread, jelly, and potatoes served, the rest of the diet being constant. The men were maintained under 24-hour daily supervision in a locked building, and during outside recreation regulated so as to provide a constant level of physical activity. All meals were prepared, measured, and served in a special dietary kitchen and the men had no opportunity to obtain other food.

The total experimental period was divided into five periods, the diet being the same in all but during periods II and IV each man ingested capsules identical in appearance providing either 8 g of arachidonic acid concentrate or 8 g of oleic acid daily. The experimental design is summarized in Table I. Venous blood

TABLE I

Experimental Design for Groups 1, 2, 3 for 70 Consecutive Days of Constant Diet

Group	I (21 days)	II (11 days)	III (14 days)	IV (10 days)	V (14 days)
1	—	A	—	C	—
2	—	C	—	A	—
3	—	C	—	C	—

NOTE: Daily dietary supplement of arachidonic acid (A) or control of oleic acid (C) provided during periods II and IV as indicated.

samples were drawn from each man on appropriately spaced occasions during the five periods.

Average characteristics of the three groups of men at the start and the changes in body weight during the five consecutive experimental periods are shown in Table II. The matching of the groups and the constancy of body weight are evident. The mean actual daily nutrient intakes for the five periods are shown in Table III.

THE ARACHIDONIC ACID PREPARATION

The arachidonic acid concentrate was generously supplied by The Upjohn Company (their preparation No. U-9519). It was prepared from liver and brain tissue, with 2 per cent each of lecithin and tocopherol added as stabilizers. Absence of *trans* double bonds was indicated by infrared spectroscopy and the nuclear magnetic resonance (NMR) spectrum. The NMR spectrum was fully in agreement with the structure of arachidonic acid and showed no extraneous bands. Ultraviolet absorption spectroscopy showed no absorption ascribable to conjugated diene, triene, or tetraene. In the assay, the octabromoarachidonic acid obtained proved to have an infrared spectrum identical with published data.[6] Assay indicated a maximum of 80 per cent arachidonic acid (by bromination) and a minimum of 40 per cent (from NMR data, assuming the material to be a mixture of arachidonic and oleic acids).

This arachidonic acid preparation was put up in opaque brown gelatin capsules, each containing 0.5 g of the preparation and estimated to contain not less than 0.25 g of arachidonic acid. The capsules were stored under refrigeration in an atmosphere of carbon dioxide. During the arachidonic supplementation period each subject ingested 16 capsules daily, thereby receiving not less than 4 g and probably about 5 g of arachidonic acid per day.

As control, capsules identical in appearance but containing 0.5 g. each of commercial grade of oleic acid were used.

TABLE II

Average Characteristics for the Three Groups of Men at the Start of the Experiment and the Changes in Average Body Weight (in kg) from the Starting Weight During the Five Experimental Periods

Group	Age	Relative weight	Δ Body weight (kg) from start				
			I	II	III	IV	V
1	47.3	95.0	−0.3	0	−0.2	−0.4	−0.5
2	46.0	95.7	−0.2	+0.1	−0.1	−0.2	+0.1
3	47.0	95.0	−0.5	+0.1	−0.1	−0.2	+0.2

NOTE: Relative weight computed from the standard U. S. Medico-Actuarial tables for height, weight, and age.

TABLE III

Mean Daily Nutrient Intakes for the Five Consecutive Dietary Periods

Nutrient	I	II	III	IV	V
Calories	3880	3970	3840	3840	3820
Proteins (g)	111.4	112.1	110.9	111.1	110.3
% calories from total fats	35.3	35.0	35.8	35.8	35.8
Saturated fatty acids (g)	70.2	70.0	69.3	69.3	69.0
Mono-ene fatty acids (g)	67.3	68.2	67.8	67.8	67.3
Poly-ene fatty acids (g)	10.4	11.2	11.2	11.1	11.2
Cholesterol (mg)	769	752	747	747	740

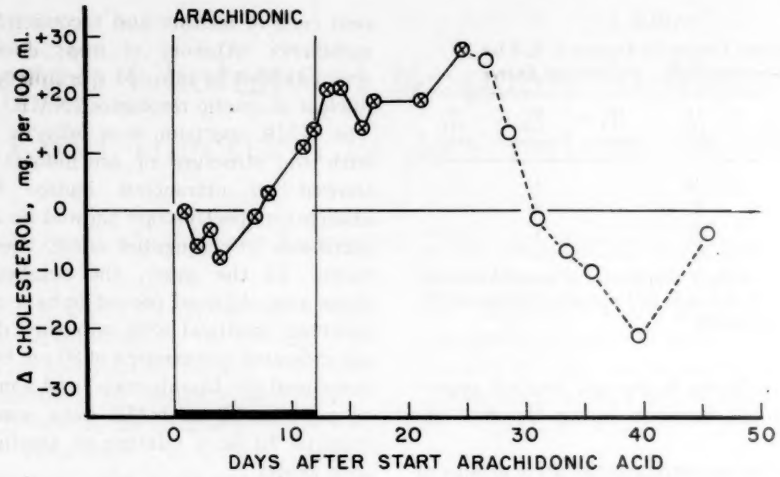

Fig. 1. Mean changes in concentration of serum total cholesterol during and after administration of arachidonic acid. Groups 1 and 2 combined (six men) until day 25; group 1 only (three men) thereafter.

METHODS

Blood serum was analyzed for total cholesterol by our modification of the method of Abell *et al.*[7] Cholesterol in the alpha and beta lipoprotein fractions, separated by fractionation with cold ethanol,[7] was measured in the same samples. All samples were analyzed in duplicate.

RESULTS

The principal experimental results in groups 1 and 2 are summarized in Figure 1. After a trivial initial decline from previous control values, the serum cholesterol rose during the last days of the administration of arachidonic acid and rose still further and continued to stay high for about two weeks after cessation of the administration.

The experimental design involved a cross over or switchback arrangement of group 1 versus group 2, i.e., arachidonic acid versus control and vice versa, with group 3 being a continuing control. It was expected that any effects of the arachidonic acid would disappear within 14 days after the cessation of its administration so that groups 1 and 3 would serve as controls when group 2 received arachidonic acid (period IV), but the unexpected persistence of an effect of the arachidonic acid after its withdrawal from the diet made it impossible to use group 1 as a control for group 2.

Fortunately, no large general time trend appeared in the controls (group 3 throughout, group 2 in periods I, II, and III). The mean values in groups 2 and 3 were 200.3, 207.3, and 202.1 in periods I, II, and III, respectively, none of these values being statistically different from their grand mean of 203.2 mg per 100 ml; the mean values in group 3, the only valid control for time trends in periods IV and V, were 191.1, 187.3, and 187.7 for periods I, IV, and V, respectively.

In the absence of a general time trend it was possible to take the average of the cholesterol values for each man during period I as his own control and to express all of his subsequent cholesterol values as deviations from that base. The averages of these individual deviations are shown in Figure 1, in which each point is the average from six blood samples. Until day 24 the six samples are from the six men in groups 1 and 2; thereafter only data for the three men in group 1 are available and each point in Figure 1 is the average of values on two consecutive days for each of the three men.

In order to obtain numbers more satisfactory for statistical analysis the data from successive time periods were grouped as shown in Table

TABLE IV

Mean Concentration of Serum Total Cholesterol (Δ Chol.), Expressed in mg per 100 ml as Difference from the Mean in Period I, at Different Times (Days) after Start of Administration of Arachidonic Acid to the Men in Groups 1 and 2

Groups 1 and 2					Group 3				
Days	Regimen	N	Δ Chol.	S.E.	Days	Regimen	N	Δ Chol.	S.E.
1–4	A	21	− 5.2	±3.3	1–11	C	21	−1.4	±2.5
7–11	A	24	+10.6	±3.6					
12–17	C	24	+19.7	±4.3	12–25	C	18	−1.3	±2.5
18–25	C	18	+24.4	±5.0					
26–33	C	15	+16.5	±4.1	26–39	C	18	−7.8	±2.1
35–39	C	15	− 6.0	±2.7					
40–49	C	15	− 9.9	±2.8	40–49	C	18	−1.2	±2.4

NOTE: Six men in groups 1 and 2 except after day 25 when only the three men in group 1 were available. Regimen A = standard diet plus capsules of arachidonic acid; Regimen C = standard diet plus capsules of oleic acid N = number of blood samples analyzed; S.E. = standard error of the mean.

IV. The average serum total cholesterol value did not change significantly during the first four days after starting the administration of arachidonic acid. During days 7 through 11 of administration, however, the cholesterol level tended to rise. This trend increased during the subsequent two weeks following cessation of administration and thereafter fell to values slightly below the means during the preadministration control period. In the control group (group 3), which received no arachidonic acid supplement at any time, there was no significant change in the serum cholesterol value at any time from period I except for a trivial fall, averaging 7.8 mg per 100 ml., during the period from day 26 to day 39.

Analyses of beta lipoprotein cholesterol as well as total cholesterol were made on the control (period I) samples from the men in groups 1 and 2 and on the samples from these same six

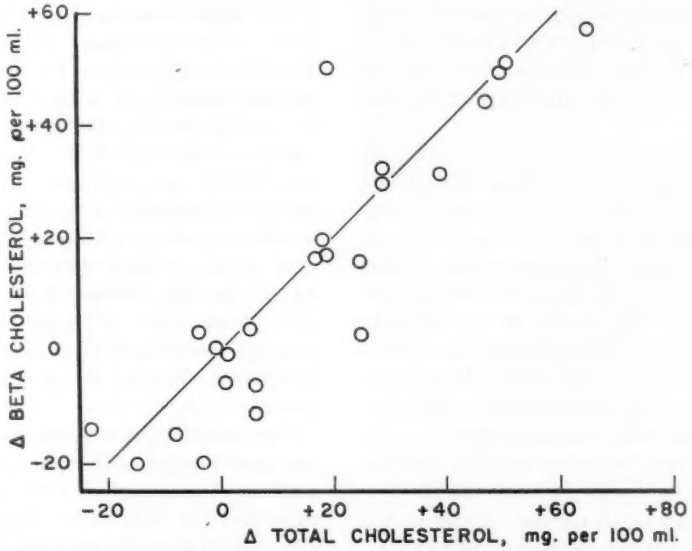

Fig. 2. Relationship between changes from individual control values for concentration of total cholesterol and beta lipoprotein cholesterol in the blood of the men in groups 1 and 2 on days 10, 11, 24, and 25 after the start of arachidonic acid administration.

men on days 10, 11, 24, and 25 after the start of the arachidonic acid administration. The grand mean difference of these $6 \times 4 = 24$ samples from the corresponding individual control averages was + 17.9 (S.E. = ±4.9) for total cholesterol and +14.6 (S.E. = ±4.8) for beta lipoprotein cholesterol, both values being in milligrams per 100 milliliters of serum. Figure 2 shows the individual values in this material. The relationship between Δ beta lipoprotein cholesterol and Δ total cholesterol is approximated reasonably well by the 45 degree linear trend line. Disregarding sign, the mean discrepancy between Δ beta and Δ total cholesterol is 7.9 mg per 100 ml, with a standard deviation of ±7.7. It appears that all the observed changes in total cholesterol may be accounted for by changes in beta lipoprotein cholesterol. This is in agreement with all other studies in which changes in serum cholesterol have been induced by dietary changes.[8,9]

DISCUSSION

The arrangements adopted in this experiment were dictated by the limited amount of arachidonic acid concentrate available. The dose used, of the order of 1.5 mg per calorie of diet or 70 mg per kg of body weight, is a large amount, many times that which could be obtained in any ordinary diet, but it is possible that a larger daily dosage would have a different effect.

Similarly, a longer period of arachidonic acid feeding might have produced effects not indicated in the present experiment. The duration of feeding chosen, 11 days, was selected because a great deal of experience in feeding various fats to men under controlled conditions indicates that much of the ultimate cholesterol response to dietary fats is usually obtained in a week to ten days, subsequent changes being merely an extension, in the same direction, of the trend usually evident in the averages of groups of subjects within a very few days.

The necessarily small number of men in the present experiment limits the security of concluding that the numerical results, as averages, apply precisely to men in general. From the present evidence it is not possible to rule out the possibility of a true slight average decline in serum cholesterol concentration in the first few days of administration of the arachidonic acid. It is clear, however, that the evidence offers no support for the idea that supplementing an ordinary diet with arachidonic acid will produce any important depression of the serum cholesterol level at any time.

Kinsell *et al.*[5] reported results in a single patient from which they concluded that arachidonic acid feeding depresses the serum cholesterol level. The preparation they used was stated to be "a phosphatide mixture derived from mammalian liver, with a fatty acid composition of 12.5 per cent of tetranoic (probably cis-arachidonic) acid, saturated acids 75 per cent, oleic acid 5 per cent, and conjugated dienes 2.4 per cent." The daily dose, for 18 days, was 75 g of this mixture, from which we estimate that the intake of arachidonic acid (or rather tetraenoic acid) was of the order of 7 g daily. The serum cholesterol data on their patient do not indicate statistically a very impressive effect and, in any case, there is no reason to believe that any effects obtained may not have been produced by the massive dosage of phosphatides rather than by the small part of the supplement accounted for by tetraenoic acid. It is interesting, also, to note that the feeding of this preparation was attended by a marked steatorrhea, with a rise in fecal fat to an average level 6.9 times the average for this patient on all other diets. No steatorrhea was observed in any of the six men studied in the present experiment, suggesting that either the patient studied by Kinsell *et al.* was peculiar or that the preparation they used produced steatorrhea because of ingredients in it other than arachidonic acid. It should be noted that, in general, steatorrhea from any cause tends to result in a decrease in the plasma cholesterol concentration.

The present data indicate that when the body has been flooded with 4 or 5 g of arachidonic acid daily for a week or so, the serum cholesterol concentration rises and remains moderately elevated for several weeks, as if the effect were produced by an excessive accumulation of arachidonic acid in the body which persists until the excess had been disposed of. Since there

is no reason to suggest that this finding does not have general validity, it is necessary to consider possible explanations.

One consequence of a glut of arachidonic acid in the body should be to inhibit the normal synthesis of arachidonic acid in the body. It is widely believed that linoleic acid is the precursor of arachidonic acid and the "linoleate is somewhat inefficiently converted to arachidonate."[10] The expectation, then, would be that flooding the body with exogenous arachidonic acid would spare the use of linoleic acid for the synthesis, thereby creating a relative surplus of linoleic acid that would be available for other uses.

Linoleic acid in the human body has three obvious uses. Besides its use in the synthesis of arachidonic acid and as a simple fuel for combustion, linoleic acid is clearly an important ingredient of lipoproteins, in which it normally comprises some 40 to 50 per cent of the fatty acids in the cholesterol esters, the remainder being largely oleic acid.[11,12] Accordingly, the release of linoleic acid from its use in arachidonic acid synthesis would be to provide an unusual abundance of a fatty acid which seems to be a preferred ingredient in the synthesis of lipoproteins. Since the liver has a large capacity to synthesize cholesterol, and there is no evidence that other ingredients of lipoproteins are normally limiting in its synthesis, the result should be to accelerate the formation of lipoprotein, including the usual and necessary complement of cholesterol in it.

This explanation of the observed phenomenon hinges on the idea that a relative surplus of linoleic acid is made available in the liver. This concept is not in conflict with the general observation that the addition of linoleic acid to the human diet tends to depress the serum cholesterol level and that of the beta lipoprotein of which it is a major constituent. It is necessary to distinguish between effects dependent on events in the liver and those that reflect events in the intestine.

From the literature[13] and from our own experiments to be reported elsewhere,[14] it appears that the major result of dietary linoleic acid on the serum cholesterol level in man is caused by the effect of dietary linoleic acid in producing an increased fecal excretion of steroids—cholesterol itself and the bile acids made from cholesterol. Apparently, linoleic acid in the diet stimulates the flow of bile, or inhibits the absorption of cholesterol and bile acids in the intestine, or both, while saturated fatty acids have the opposite effect. The indications are that the rate of cholesterol synthesis in the liver is actually increased on linoleic acid feeding, and decreased on feeding saturated fatty acids, but that these changes in the rate of synthesis are insufficient to compensate fully for the changes in the removal, via the feces, of cholesterol and the steroids derived from it. In other words, the primary effects of the fatty acids in the diet would seem to be on the bile flow and reabsorption of bile constituents in the intestine, changes in the synthesis in the liver being perhaps a secondary and imperfect homeostatic response.

There is some evidence that there is competition for the use of linoleic acid for synthetic purposes in the liver. When animals are maintained on a diet severely restricted in linoleic acid, the addition of large amounts of cholesterol to the diet precipitates or enhances the development of signs of essential fatty acid deficiency.[15] The explanation may be that there is competition for the linoleic acid for the synthesis of arachidonic acid on the one hand and for the synthesis of plasma lipoprotein on the other; an overabundance of exogenous cholesterol would favor the route of use to make lipoproteins. It is interesting to note also that on extremely low fat diets, in which the supply of linoleic acid is necessarily very small, the serum cholesterol, and presumably also the associated beta lipoproteins, does not rise but may fall to very low levels.[16] A deficiency of linoleic acid for the esterification of cholesterol to go into lipoproteins would seem to be a reasonable explanation; it is unnecessary to suggest that there is any defect in the ability of liver to synthesize cholesterol in this situation.

SUMMARY

In a rigidly controlled experiment with the subjects on a constant diet of two alternating daily menus, six middle-aged men ingested capsules containing a concentrate of arachi-

donic acid or, during control periods, of oleic acid. Three other men, matched in age and other characteristics with those in the experimental group, subsisted on the same diet but ingested only oleic acid capsules. The arachidonic acid dosage was 4 to 5 g per man per day for 11 days.

The concentration of total cholesterol in the blood serum showed no significant change during the first few days of supplementation with arachidonic acid but thereafter it tended to rise, and it remained elevated for several weeks following the withdrawal of the arachidonic acid supplement before returning to approximately the presupplement level. The changes in the serum total cholesterol concentration were accounted for by changes in the cholesterol in the beta lipoprotein fraction of the serum. The significance of these findings is discussed.

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Mrs. Helen Williams supervised the diet preparation and measurements. Mrs. Nedra Foster supervised the blood cholesterol measurements.

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The Anti-Coronary Club; Including a Discussion of the Effects of a Prudent Diet on the Serum Cholesterol Level of Middle-Aged Men

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THE Diet and Coronary Heart Disease Study Project (usually known as the Anti-Coronary Club) originated in an Executive Order of the Commissioner of Health, dated February 19, 1957, in which she appointed a Task Force to develop and supervise a study of the relationship between diet and coronary heart disease.

The first few weeks were spent in devising administrative procedures, the recruitment of personnel and in detailed planning of the study. This plan was implemented May 23, 1957, when the Board of Estimate approved an appropriation for the study.[§]

The first volunteers were received on June 3, 1957. Two years later the membership reached its projected goal of 600 males, aged 20 to 59 years, of whom approximately 60 per cent are in the highly susceptible 50 to 59 year age group, 20 per cent in the 40 to 49 year age group and the remaining 20 per cent in the 20 to 39 year age group. The younger groups are included for comparison with the 40 to 59 year age groups.

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BACKGROUND OF THE STUDY

By the end of 1956 certain findings concerning diet and coronary heart disease had been sufficiently established[1] to instill a fair degree of confidence that a public health approach to the prevention of coronary heart disease through diet could now be attempted. These may be stated briefly as follows:

(1) In most countries of the Western world, coronary heart disease had become the leading cause of death in men 30 years of age and older. Of all these Western countries it was highest in the United States;[2] in the United States it was highest in New York State[3] and, in 1955, it was about 10 per cent higher in New York City than the rest of the State.[4] For example, in men 50 to 59 years of age in New York City in 1957 the death rate for arteriosclerotic heart disease (ISC 420) was 616 per 100,000. Furthermore, it had risen many fold since 1920. Much of this increase has been factitious because of changes in procedures and classifications used for recording causes of death, increasing age of the population and varying degrees of medical sophistication.[5–7] This latter factor includes such considerations as changing levels of medical training and experience, broadening of the concept of coronary heart disease, better diagnostic methods, and increasing fashion of this diagnosis.

However, whether this increase is wholly or partially factitious is irrelevant to the argument because the major problem is not whether the mortality rate from coronary heart disease has increased significantly from some base period[7] (which may or may not have been adequately measured) but why it is at its present high levels in affluent Western countries and much less in certain other countries and communities.

(2) Epidemiologic associations between total fat consumption and death rates for coronary heart disease in various countries of the world indicate a close relationship, an association made much more significant when the death rates are compared with the consumption of saturated types of fat rather than total fat.[1,7,8] Although other dietary components (e.g., animal protein and total calories)[7,9] show a close association, that with saturated type of dietary fat is the highest.[7] This association of saturated types of fat is an explanation for the fact that in 1954–1955 total fat consumption in Norway, Denmark, and Sweden was as high as it was in the United States or England but death rates for coronary heart disease were only one-third to one-half that of these latter countries.[7,8]

(3) In all countries and communities in which these determinations have been made, in which the middle-aged males had an average blood cholesterol level over 220 mg per 100 ml, a problem of coronary heart disease exists[10] (death rates above 350 per 100,000).[7] In communities in which the average serum cholesterol level in this age group was below 200 mg per 100 ml, there is no major problem of coronary heart disease. Even in our own country, the cooperative study[11] involving over 4,000 men aged 40 to 59 years showed that, within a period of one to two years, men whose cholesterol levels were in the top third of the distribution had about three times the number of new events as did the men whose cholesterol levels fell in the bottom third. Furthermore, the Framingham Study[12] has recently shown that males whose blood cholesterol level was above 260 mg per 100 ml had, over a four-year period, six times the incidence of new events, compared with those whose cholesterol was less than 225 mg per 100 ml (80 per thousand vs. 13 per thousand).

(4) It had been established by Kinsell, *et al.*[13] and confirmed by Ahrens,[14] Beveridge,[15] Malmros,[16] and their associates that, under the conditions of the metabolism ward or formula diets in trained individuals, the substitution of oils high in polyunsaturated fatty acids (e.g., corn, cottonseed, marine, safflower, soybean, sunflower) for the dietary saturated fats would lower the blood cholesterol.

Considering the scientific background that had been developed by 1957, and taking into consideration our facilities (some of which are not duplicated by other major health departments), our personnel and our experience in motivating obese subjects in following prescribed diets over long periods of time, it was decided to make our over-all objective a dietary approach to the prevention of coronary heart disease. This over-all objective was to be reached by the following major steps:

(1) Develop practical diets for use by the general public to replace metabolism ward and formula diets. These diets must be nutritionally adequate, meeting the specific nutrient requirements of the Recommended Dietary Allowances of the Food and Nutrition Board of the National Research Council;[17] must be palatable; and consist of acceptable American foods available in every community. In addition, the dietary pattern must be consistent with the normal diet pattern recommended by the U. S. Department of Agriculture in their Home Economics Research Report No. 3.[18]

(2) Demonstrate that if persons in their usual environment can be induced to modify their diets over a long period of time, their blood lipids, as measured by the cholesterol-lipoprotein system, will be favorably influenced. It remains to be proved, however, that a significant number of persons free-living and consuming common American foods can be so induced. This is a clear challenge to our ability to motivate and educate so that a change in the diet of free-living persons will result.

(3) Demonstrate whether a favorable change in the cholesterol-lipoprotein system produced and maintained by diet is associated

in fact with a favorable change in morbidity and mortality from coronary heart disease, particularly in men under 65 years of age.

PROCEDURES OF THE ANTI-CORONARY CLUB

The first subjects were obtained in response to a short release carried in most of the New York City newspapers. This release announced the formation of such a unit in the Department of Health and requested male volunteers from all segments of the population who would be willing to follow our dietary recommendations over a long period of time. This was repeated a month later. The response to these announcements was so good that further publicity has not been necessary. Telephone and written inquiries were answered by a "Fact Sheet" and an application blank requesting certain pertinent data that would be necessary for the proper selection of subjects. The main criteria for selection were that the subject should be a male adult, under 60 years of age; that he reside in New York City; that he have no other serious disease which might shortly terminate his life, and that he be physically capable and have sufficient time available during ordinary working hours to participate in the study. A previous history of coronary heart disease, obesity, hypertension, gout, low metabolic rate, diabetes, or hyperthyroidism did not exclude a volunteer. The acceptable subjects were then invited to an orientation lecture at which the purpose and functions of the Anti-Coronary Club were explained in greater detail. It was pointed out to them at this time that the Club was a research unit, not a service clinic, that they would remain under the care of their private physician and that the two would cooperate and exchange records, There has been remarkable cooperation with the private physicians.

It was further pointed out to the subjects in the 50 to 59 year age group that they were expected to remain in the study and follow the recommended diet pattern until they reached 65 years of age. Those in the younger age group (20 to 49 years) were expected to remain in the study for a five- to 10-year period. Those who chose to become volunteers (usually about 75 to 85 per cent of those who appeared at the orientation lecture) were then scheduled for work-up by appointment at a mutually convenient time. This work-up consisted of a thorough medical history and physical examination; roentgenograms of the heart, abdominal aorta, iliac and tibial arteries; serologic complete blood count; glucose tolerance test and other routine blood chemical determinations. When these were determined and no disease found other than those mentioned previously, the subject was scheduled for a detailed diet history obtained by recall and diet records. This dietary history is sufficiently detailed so as to give not only the proximate composition of the diet in terms of carbohydrate, fat, protein, minerals, and vitamins, but also an estimate of the amount and quality of fats consumed. This latter detail enabled us to classify the estimated amounts of the various types of fatty acids: saturated, monounsaturated and polyunsaturated. During this time the control blood cholesterol levels were determined, in duplicate, by the Anderson-Keys[19] modification of Abell's method,[20] repeated at frequent intervals during a control period of one to three months, while the patient continued to follow his usual diet. We have called the average of these cholesterol determinations the *benchmark* level.*

When the work-up was completed and the benchmark level determined, the patient was seen by a panel of physicians consisting of one or both of the senior physicians, one of the examining physicians and one of the graduate students assigned to this study. This system of a panel discussion seemed more satisfactory as a motivating reinforcement than having the patient seen by only one of the panel members. At this panel discussion, the findings pertaining to the particular subject were discussed and the type of diet pattern prescribed. In general, the type of diet pattern prescribed depended upon whether or not the subject was overweight.

* The technical error of our laboratory determination on the basis of over 100 blind duplicate samples is 8.3 mg/100 ml. This represents an error of 3.3 per cent. A change of 10 per cent in the individual subject, three times the technical error, has been considered significant.

Normal weight was determined by considering a combination of comparison of the present weight of the subject with standards of height and frame, skin caliper determinations, ruler test[21] and appearance of the subject. If he was of normal weight, a prudent diet pattern* (designated in this project as "the prudent diet") was prescribed and the subject was sent to one of the nutritionists for detailed instructions to suit the individual's calorie needs and food preferences. Thus an individual diet was prescribed for each subject. The instructions for the prudent diet pattern were as follows:

(1) Consume adequate amounts of high grade protein at each meal. The sources for these include: cottage-type cheese, fat-free milk, chicken, turkey, veal, leaner cuts of beef, mutton, lamb, and pork (with the visible fat removed), fish, seafood, and egg whites.

Remember there are 21 meals each week. Therefore, from this list, include fresh or canned fish or seafood at least five times each week. Do not be limited to the leaner fish but include fat fish as well as they are good sources of polyunsaturated fatty acids. Bake, roast, broil, or boil meats and poultry. Fish may be fried as instructed. Serve beef, lamb, mutton, and veal medium to well done: when rare, less fat drains off. When fat is required in food preparation, use a high linoleic acid oil† or an 80 per cent unaltered high linoleic acid margarine or cooking fat. Use veal or fowl four meals each week or oftener. Use beef, lamb, mutton, or pork not oftener than four times each week. For the other eight meals each week use cottage-type cheese or egg whites. Unless instructed otherwise, you may include four egg yolks each week. You may also use whole milk for coffee but otherwise restrict the use of milk to nonfat varieties such as fresh or reconstituted skim milk, fat-free buttermilk, or evaporated skim milk. When instructed, you may add an emulsion of corn oil to your skim milk.

(2) Consume a minimum of 1 ounce of corn oil, or its equivalent, at the table each day exclusive of that used in food preparation. This may be added to milk, cereals, or desserts, or an 80 per cent unaltered corn oil margarine used as a table spread. When fats are desired in cooking or baking, use corn oil or a specially prepared equivalent.

(3) Do not eat the following except under unusual circumstances: butter, ordinary (partially hydrogenated) margarine and shortenings, lard, or cream or foods containing them in large amounts such as most cakes and pastries. In place of butter and margarine, use an 80 per cent unaltered corn oil margarine;‡ in place of shortening use either corn oil or a specially prepared 80 per cent unaltered corn oil margarine;‡ in place of cream use an emulsion of corn oil.§

(4) Balance your diet by consuming adequate amounts of bread, cereals, nuts, vegetables, and fruits.

TABLE I

Proximate Composition of the Prudent Diet* Pattern as Followed in Practice

Nutrient	G	Calories (%)
Protein	130–150	23–26
Carbohydrate	225–280	42–45
Fat	66–97	30–33
Saturated fatty acids	19–21	7–8
Oleic acid	25–26	8–9
Polyunsaturated fatty acids	20–33	9–11

* Contains 2,000 to 2,700 calories.

* Prudent—exercising sound judgment, characterized by practical wisdom or discretion (Funk and Wagnalls Practical Standard Dictionary).

† Although other high linoleic acid oils could be used (e.g., sunflower, safflower, soybean, cottonseed) in this study we have used corn oil exclusively in order to control the fat component of the diet as closely as possible, and because corn oil is available in every grocery store and labeled as such, because taste-test indicated its wide acceptability and because it possesses a relatively high content of vegetable sterols not always found in other oils. This latter factor is considered important by some investigators.[22,23]

‡ Kindly supplied us by the Pitman-Moore Co., Indianapolis, Ind., as Emdee Margarine.

§ Kindly supplied us as a flavored pharmaceutical emulsion by the Gray Pharmaceutical Co. of Newton, Massachusetts, as Atheroxine; and by The Upjohn Co. of Kalamazoo, Michigan, as Lipomul; and as an unflavored emulsion of corn oil in skim milk through the cooperation of the Dannon Milk Products Co. of Long Island City, New York.

The dietary records following dietary instruction of the males in the 50 to 59 year age group indicated that this diet pattern provides in practice the composition shown in Table I. These were calculated from food composition tables.

During the first month on the prudent diet, the patient, often accompanied by his wife, returned at frequent intervals for further diet instructions. Both were encouraged to telephone the nutritionist at any time a dietary problem arose. After the first month, the patient was encouraged to consult the nutritionist as often as desired. The subjects also returned every ten weeks for meetings with the panel of physicians, or oftener at the patient's request if some particular problem was involved. The patient returned for blood cholesterol determinations at monthly intervals. At yearly intervals the patient returned for a complete medical and electrocardiographic follow-up.

If the subject was overweight, he was placed on the weight reducing type of prudent diet. This reducing diet will not be described here but in greater detail elsewhere at another time. In general, it consisted of the same food pattern as described heretofore except that no fat of any kind was added to cooking or used at the table. This made the average diet for

TABLE II

Monthly Serum Cholesterol Determinations
Weight and Coronary Heart Disease Condition of 79 Subjects
Six Months Adherence to Prudent Diet

Serum cholesterol (mg per 100 ml) Month							Coronary disease*	Weight	
B	1	2	3	4	5	6		Base	6 Mo.
350	318	320	313	306	300	288	NC	168	166
346	342	320	298	276	280	287	NC	144	145
339	304	268	268	269	270	273	NC	160	154
325	313	300	307	335	317	300	NC	150	150
318	303	295	273	272	230	240	NC	149	147
318	230	250	244	237	230	230	NC	142	139
308	280	243	285	240	233	242	NC	158	156
308	305	290	289	288	311	270	NC	171	161
307	287	267	263	260	260	260	NC	188	185
305	271	275	270	284	295	245	C	134	134
304	300	300	302	312	316	320	C	142	138
300	300	250	250	269	270	272	NC	154	153
299	240	250	275	243	243	243	NC	161	160
296	305	300	295	290	270	290	NC	162	164
294	272	250	250	250	235	215	NC	152	149
293	265	255	250	245	251	258	NC	188	184

(TABLE II *Continued*)

287	271	261	251	265	225	240	C	148	150
282	245	257	232	245	245	250	NC	146	140
282	260	239	248	251	255	257	NC	154	162
279	247	215	217	219	220	220	C	127	117
279	221	208	219	230	223	228	C	155	148
278	190	205	209	213	212	210	NC	152	149
276	217	222	223	224	225	213	NC	152	148
274	265	252	235	222	227	232	C	148	147
273	264	266	268	268	269	294	C	143	142
273	260	247	248	249	250	243	NC	181	186
271	245	211	213	219	225	231	NC	153	147
270	240	210	215	220	225	183	NC	182	170
265	247	257	269	277	287	268	NC	158	160
264	220	250	242	233	224	215	NC	184	175
262	243	214	225	236	247	258	NC	139	138
262	253	270	248	215	220	226	NC	147	149
260	261	262	256	250	252	250	NC	180	181
260	220	202	214	197	187	183	C	151	148
259	258	257	218	226	204	234	C	139	138
258	225	225	192	217	212	206	C	122	120
256	330	315	307	250	270	290	NC	159	147
255	250	253	238	213	225	235	NC	147	140
254	240	225	216	208	200	215	NC	125	125
254	275	272	269	266	263	260	NC	144	146
253	253	253	245	237	233	215	NC	181	184
252	227	217	207	197	201	205	NC	136	132
251	250	277	250	250	235	243	C	140	143
247	240	243	223	245	235	235	C	142	143
243	227	207	220	225	197	212	NC	148	145
239	219	200	202	204	205	198	NC	139	136
237	257	257	261	265	225	196	C	159	158
236	233	235	210	197	210	205	NC	164	162
235	229	223	223	224	250	236	C	156	154
234	222	223	225	237	205	205	C	163	166
230	218	207	208	210	160	185	NC	169	168
230	210	178	180	183	194	205	NC	140	140
228	237	243	217	216	216	215	C	156	156
227	185	178	181	181	188	171	NC	163	148
225	215	204	201	198	195	185	NC	173	172
222	225	220	215	200	212	225	NC	162	165
220	205	218	225	223	214	205	NC	141	143
214	206	198	191	200	203	205	C	114	118
213	250	237	212	195	200	205	C	171	170
212	192	198	208	218	218	195	C	166	164
211	225	233	195	193	200	185	NC	148	146
211	205	200	200	200	200	209	NC	178	179
210	217	223	208	220	218	168	C	135	135
210	212	297	204	210	201	193	NC	156	153
210	200	193	193	193	177	193	NC	215†	211
208	193	197	198	225	195	178	C	158	161
208	220	215	215	190	207	215	NC	157	159
200	198	187	202	183	160	180	NC	125	133
200	193	207	195	180	179	178	NC	147	147
199	212	223	203	213	222	226	NC	144	145
198	187	194	200	212	192	205	NC	156	156
196	206	186	193	200	193	215	C	153	155
196	203	203	194	195	194	198	NC	144	143
195	200	163	159	155	165	176	C	170	172
184	166	175	165	200	200	160	C	157	156
184	202	195	195	194	190	184	NC	164	163
182	162	140	163	157	150	156	C	130	135
180	168	175	133	120	130	120	NC	154	153
177	210	200	193	190	180	170	NC	158	157

* NC = no coronary heart disease using the criteria of the New York Heart Association. C = coronary heart disease using the criteria of the New York Heart Association.

† This subject had a relative weight of 115 per cent for his height and frame which would make him borderline between normal and overweight. However skin thickness at the angle of the scapula, over the lower ribs at the mid-axillary line, and the mid-arm were all normal, the abdomen did not protrude, and the general appearance was that of normal weight.

this group of men about 1,600 calories. On reaching desired weight, the patient continued on the same diet plus the addition of cooking and table fats, of the types used in the previously described prudent diet, sufficient to maintain the desired weight. This converted the reducing diet to the prudent diet.

This is neither the time nor the place to give the detailed results of the study to date. Obviously, since this study has been under way for less than two years and the subjects have been added *ad seratim*, sufficient time has not yet elapsed for this study to give even a preliminary answer as to whether or not changes in the serum lipoprotein system will influence the morbidity and mortality rates from coronary heart disease. It is possible, however, to give a preliminary report on the effect of a prudent diet on the cholesterol level of 79 men, aged 50 to 59 years, of normal weight when they came to us, who have followed the prudent diet pattern for six months or longer.

METHOD

Benchmark cholesterol levels either in the fasting state or after a fat-free breakfast were determined for 220 men aged 50 to 59 years while on their usual diet. The distribution of the serum cholesterol levels in these 220 men showed that one-third were in the range 270 mg per 100 ml or more, one-third less than 230 mg per 100 ml and the remaining third in between. This distribution is consistent with that found by the cooperative study.[11] As of June 30, 1958, 86 normal weight men had been placed on the prudent diet. By the end of 1958, 79 of these men had completed a period of at least six months' adherence to the prudent diet. The seven subjects who did not complete six months of observation represented 8 per cent of the starting group: four failed because of the time factor involved in the follow-up studies; two could not adjust to the diet pattern; one dropped out on advice of his private physician.

The group of 79 men was divided according to the tertiles into which they fell determined on the basis of the benchmark cholesterol averages of the total 220 men. Twenty-eight men fell into the upper tertile (270 mg per 100 ml or more), 24 in the middle group, and 27 in the lower group (less than 230 mg per 100 ml). Twenty-five of the 79 men (31.6 per cent) had clinical evidence of coronary artery disease. This is comparable with the coronary experience of the total group of 220 men in the same age group in which 33 per cent had such clinical evidence.

RESULTS

Table II shows the successive individual blood cholesterol determinations arranged in order of the magnitude of benchmark level at monthly intervals for six months, the original

TABLE III

Average Serum Cholesterol by Month

Tertile	No.	Months After						
		B*	1	2	3	4	5	6
		Average Serum Cholesterol (mg per 100 ml)						
High	28	297.6	270.0	258.1	257.5	257.2	254.0	251.7
Middle	24	249.8	242.0	238.5	231.1	227.6	222.5	224.6
Low	27	204.4	202.6	199.4	194.4	194.6	192.5	189.4
Total	79	251.3	238.4	232.1	227.9	226.8	223.4	222.0
		Per Cent of Benchmark Level						
High	28	100.0	90.7	86.7	86.5	86.4	85.3	84.6
Middle	24	100.0	96.9	95.5	92.5	91.1	89.1	89.9
Low	27	100.0	99.1	97.6	95.1	95.2	94.2	92.7
Total	79	100.0	94.9	92.4	90.7	90.3	88.9	88.3

* B—Benchmark (control) level.

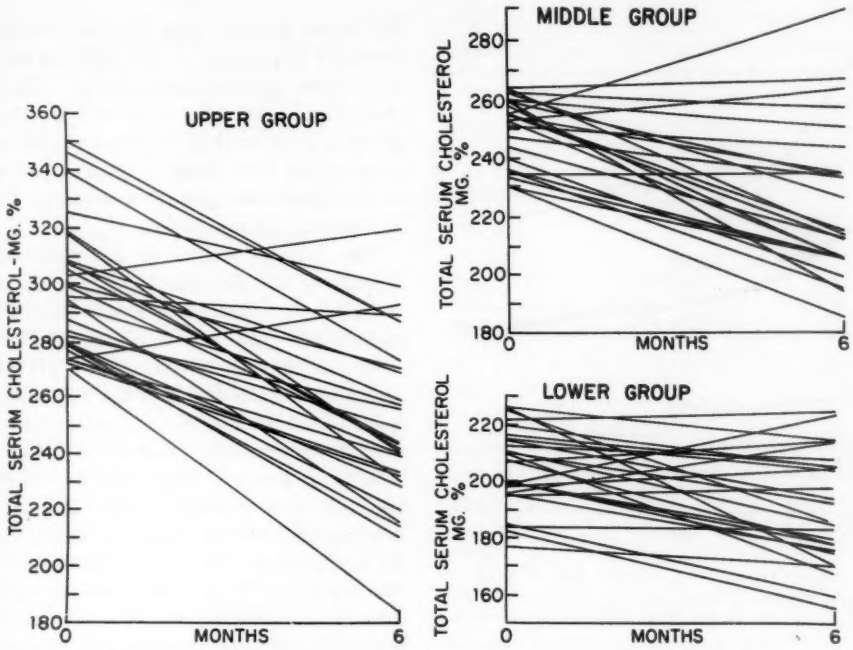

Fig. 1. Individual serum cholesterol response of 79 men of normal weight aged 50 to 59 years to six months' adherence to prudent diet.

weight and the weight at six months, and original diagnosis as to presence or absence of clinical coronary heart disease.

The average benchmark cholesterol level of all 79 men was 251 mg per 100 ml; after six months on the diet (as shown in Fig. 1 and Table III) this average had fallen to 222 mg per 100 ml with about 75 per cent of the six-month-fall occurring in the first three months. These changes between the benchmark levels and the six month levels are shown graphically in Figure 2. Initially, the cholesterol averages of the three tertiles were 298, 250, and 204 mg per 100 ml, respectively. After six months on the prudent diet, these levels were 252, 225, and 189 mg per 100 ml (Fig. 1 and Table IV) decreases of 46, 25, and 15 mg per 100 ml, respectively. These represent decreases of 15.4. 10.1 and 7.3 per cent, respectively (Table III). The decreases in the serum cholesterol from the benchmark levels in the group as a whole and in the individual tertiles were each statistically significant beyond the 1 per cent level (Table IV).

The individual response to this diet seems to depend roughly upon the benchmark cholesterol level. This is illustrated in Figure 2 and Table V. Using a change of more than plus or minus

TABLE IV

Average Serum Cholesterol Levels by Tertile Before and After 6 Months on Prudent Diet

Tertile	Benchmark cholesterol range	Total Patients	$\bar{X}$			σ			t	P
			Bench-mark	6 Mo.	D	Bench-mark	6 Mo.	D		
High	270+	28	297.6	251.7	45.9	23.1	30.9	7.3	6.23	<0.01
Middle	230–269	24	249.8	224.6	25.2	11.9	26.0	5.8	4.34	<0.01
Low	229–	27	204.4	189.4	15.0	14.9	23.6	5.4	2.78	<0.01
Total	177–350	79	251.3	222.0	29.3	42.2	37.8	6.4	4.53	<0.01

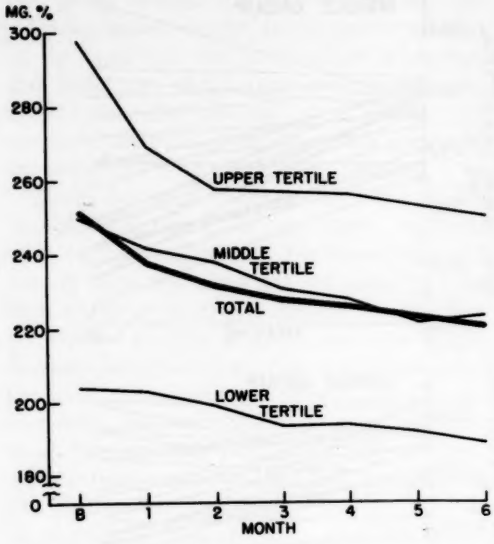

Fig. 2. Serum cholesterol averages for 79 men of normal weight aged 50 to 59 years on prudent diet for six months.

10 per cent from the benchmark level after six months as a criterion, it can be noted that the serum cholesterol level fell in 78 per cent of the upper group. This compared with 58 per cent and 30 per cent of such cases in the middle and lower groups respectively. The serum cholesterol level of no subject in the upper group rose more than 10 per cent although two approached this figure. One person in each of the other two groups rose above this limit and several approached it.

The average weight of the group after six months on the diet was 153.0 pounds, a loss of 1.3 pounds. Four individuals gained 5 to 8 pounds while ten lost 5 to 15 pounds (Table IV).

It can be noted from Table VII that the serum cholesterol levels of the 25 patients with evidence of coronary heart disease had a lower average benchmark cholesterol (240.9 versus 256.1 mg per 100 ml) than the 54 subjects without such evidence. This is true for the benchmark levels of both the upper and middle age groups. Our records indicate that patients coming to us with overt coronary heart disease had already been placed on some sort of restricted fat and/or calorie intake by their doctors for months to several years before participating in this study.

The average benchmark fat intake of the

TABLE V

Reaction of Serum Cholesterol Level to Prudent Diet (Six Months)

Tertile	Benchmark cholesterol range	No.				Per cent			
		Total	Fell*	Remained	Rose†	Total	Fell*	Remained	Rose†
High	270+	28	22	6	0	35.5	78.5	21.5	0
Middle	230–269	24	14	9	1	30.4	58.4	37.6	4.0
Low	229–	27	18	18	1	34.1	29.6	66.7	3.7
Total	177–350	79	44	33	2	100.0	55.7	41.6	2.7

* Fell more than 10 per cent of benchmark level.
† Rose more than 10 per cent of benchmark level.

TABLE VI

Weight by Coronary Heart Disease Condition

Tertile	Heart Disease Number			Weight (lb)						5 lb or more					
				Control			6 months			Gained			Lost		
	T	C	NC	T	C	NC	T	C	NC	T	C	NC	T	C	NC
High	28	7	21	155.9	142.4	160.6	153.3	139.4	158.1	2	0	2	6	1	5
Middle	24	8	16	151.3	146.5	153.8	149.9	146.3	151.8	0	0	0	3	0	3
Low	27	10	17	155.5	151.0	158.8	155.7	152.2	158.1	2	1	1	1	0	1
Total	79	25	54	154.3	147.1	157.9	153.0	146.7	156.1	4	1	3	10	1	9

NOTE: T—total; C—coronary heart disease; NC—no coronary disease using criteria of the New York Heart Association.

TABLE VII

Average Serum Cholesterol by Coronary Heart Disease Condition Before and After Six Months on Prudent Diet

Tertile	Benchmark levels			6-month levels			No.		
	T	C	NC	T	C	NC	T	C	NC
High	297.6	285.9	301.6	251.7	254.1	251.5	28	7	21
Middle	249.8	247.6	250.9	224.6	218.5	227.6	24	8	16
Low	204.4	204.2	204.5	189.4	187.3	190.6	27	10	17
Total	251.3	240.9	256.1	222.0	216.0	225.2	79	25	54

25 subjects with coronary heart disease was 30 per cent while that of the 54 subjects without coronary heart disease was 36 per cent. These differences are significant beyond the 0.01 level. Table VII also shows the effects of the diet on the serum cholesterol of subjects with and without coronary artery disease. Regardless of these findings, however, those patients with positive diagnosis of coronary artery disease using the criteria of the New York Heart Association did not respond differently than did those with no coronary artery disease but similar benchmark cholesterol levels.

DISCUSSION

The 79 men of normal weight, aged 50 to 59 years, in this series, had an average "benchmark" total serum cholesterol level of 251 mg per 100 ml. The cooperative study[11] showed an average serum cholesterol of 242 mg per 100 ml in over four thousand clinically normal males, aged 40 to 59 years, and 243 mg per 100 ml in the 1,464 men in the 50 to 59 year age group. Thus, the mean benchmark cholesterol level of our 79 males was 3 per cent higher than that of a similar age group in the cooperative study.

There may be bias in some respects inherent in the group with which our study is concerned. Our subjects are all volunteers and, to the extent that they are sufficiently interested in the subject of cholesterol control to donate a substantial amount of time and effort to submit to comprehensive medical and laboratory examinations as well as to dietary instruction, they probably do not represent the present attitude of this age group of the male population at large. Although this bias may affect the motivation, and through it, the ability of volunteer subjects to adhere to a diet over a long period of time under free-living conditions, it is difficult for us to imagine how this type of bias could influence the response of the blood cholesterol level to dietary change.

Our study demonstrates that the prudent diet as defined possesses a significant cholesterol-lowering effect in most subjects and is one with a high degree of acceptability. It differs from the others reported in several aspects. Our subjects were free-living and working men who took their meals at home or in restaurants and ate foods readily obtainable. Although Brown and Page's study[24] deals with ambulatory subjects, their subjects and patients either received all their meals at the diet kitchen or were trained for 11 to 21 days in the diet kitchen before continuing at home. Further, 15 of their 16 patients were hyperlipemic, representing a single type of patient. The dietary pattern of our subjects provided at least 130 g. of protein, 50 per cent more than the 86 g. provided by the vegetable oil diet of Brown and Page.[24] Our dietary pattern provided somewhat less carbohydrate and about half as much vegetable oil. The saturated fat content of both diets was low and about similar, and both were effective in lowering the serum cholesterol.

Several groups of investigators have used either supplements of straight vegetable seed oils or their emulsions as a means of lowering blood cholesterol, with little or no dietary reeducation. Meltzer, Bockman, and Berryman[25] studied the effect of a pharmaceutical emulsion of safflower oil, plus sitosterol, plus pyridoxine, plus vitamin E on the blood cholesterol level of 28 patients aged 36 to 53 years with hypercholesterolemia and myocardial infarction. This emulsion was administered for six

months as a dietary supplement, with instructions that the patients maintain their previous fat intake. The over-all change after six months was a significant decline in serum cholesterol levels for all ten patients consuming less than 50 g. dietary fat daily, for seven of eight patients consuming 50 to 100 g. dietary fat daily, and for seven of ten patients consuming 100 to 150 g. dietary fat daily.

Gordon and Brock's[26] group of ten men were aged 33 to 59 years, eight of whom received 50 g. of sunflower seed oil added daily as a supplement to their regular diet. One received 37 g. of a pharmaceutical preparation while the remaining subject received 75 g. of either sunflower seed oil or pilchard oil. These authors reported a significant fall in the serum cholesterol level in eight of their ten subjects within six months by this method. It should be noted, however, that two of their subjects dropped out before completion of the study because of excessive weight gain and two of the ten subjects completing the six months' period gained 12 and 15 pounds, respectively. In our 79 subjects, only four gained 5 pounds or more (5 pounds, 5 pounds, 8 pounds, and 8 pounds, respectively) and, as a group, the average weight decreased 1.3 pounds whereas the Gordon and Brock group gained an average of 3.6 pounds during the fat supplement period. As pointed out by these authors, many of their patients did not continue on their usual previous diet as evidenced by the fact that they did not gain weight as expected and some even lost weight. The finding of Gordon and Brock[26] that the simple addition of polyunsaturated oil to the regular diet has a hypocholesterolemic effect was not found by Perkins, Wright, and Gatje[27] who reported no significant effect on the cholesterol when an emulsion containing 48 cc of safflower oil was added daily to the regular diet of 24 medical students, aged 22 to 26, for six to seven weeks.

McCann and her associates[28] followed a group of 20 faculty members, aged 37 to 65, who received daily, for four weeks, 8 ounces of a peanut oil emulsion supplying 960 calories and containing 36 per cent peanut oil and 4 per cent coconut oil. Their subjects were instructed to keep their weight constant, which necessitated omission of some food, usually the visible fat, from their regular diet. Twelve persons in this group showed a fall in serum cholesterol of more than 10 per cent from the original level. Later, 10 of the original 20 men completed a further four-week study in which they took three tablespoonfuls daily of a 50 per cent safflower oil emulsion. Of these ten men, six showed a decrease of 10 per cent or more in serum cholesterol level. McCann *et al.* noted that it is highly improbable that such subjects as theirs who had not suffered a myocardial infarction would continue on this type of diet pattern over a long period of time.

However, Tobian and Tuna[29] studied 23 patients with coronary atherosclerosis with an average blood cholesterol level of 271 mg per 100 ml. Their patients were instructed to ingest 1 to $1^1/_2$ ounces of corn oil before each meal and to use corn oil in the preparation of food. Eighteen of these patients were instructed ". . .to avoid butter fat, margarine, various hydrogenated vegetable oil preparations, and more than one egg a day." The other five were given no dietary restrictions. The drop in cholesterol was significant in both groups and maintained over a period of one year. Tobian and Tuna, however, made no mention of the number of patients starting in their study.

We have followed the tenet that in order to keep the weight stable, the substitution of one quality of dietary fat must be made for another. Further, this may limit the need for larger amounts of vegetable oil *per se* since the cholesterol-elevating propensity of 1 g of saturated fat seems to be balanced by the cholesterol-lowering action of 2 or 3 g of unsaturated fat.[22] In this regard, Kinsell also states,[30] "If lowering the plasma cholesterol level is to be achieved by the use of polyunsaturated fats, it is necessary to modify the entire diet."

The capacity of the prudent diet to lower cholesterol levels is not limited to those with hypercholesterolemia for we have found significant falls in cholesterol levels in each tertile. Of 86 men who started on the prudent diet in this period, 79 have followed it for six months or longer. This would indicate a

high degree of acceptance by our subjects, an important consideration, as neither a formula diet nor a low protein diet nor a low fat diet (less than 20 per cent of total calories) is destined for popular acceptance as a preventive measure.

Effective and acceptable as it is, we do not yet know whether the prudent diet pattern is the optimal that can be devised for a public health program.

SUMMARY AND CONCLUSIONS

The average control (benchmark) cholesterol level of 79 men of normal weight, aged 50 to 59 years, was found to be 251 mg per 100 ml. When distributed into previously established tertiles on the basis of their cholesterol levels, 28 men fell in the highest third (270 mg per 100 ml and over), 24 in the middle and 27 fell, into the lowest third (under 230 mg per 100 ml).

They were placed on a diet of 2,000 to 2,700 calories, with fat 66 to 97 g (30 to 33 per cent of total calories), protein 130 to 150 g, and carbohydrate 225 to 280 g. Predominantly saturated types of fat yielding 7 to 8 per cent of the total calories as saturated fatty acids were limited to not more than 15 per cent of total calories. The balance of fat (up to a total of 33 per cent of total calories) was derived from unsaturated types comprising 1 to $1^1/_2$ ounces of corn oil or its equivalent and fats from fish, grain and vegetables with total amount of polyunsaturated fatty acids comprising 9 to 11 per cent of total calories.

After six months on our diet, the group cholesterol average had fallen from 251 to 222 mg per 100 ml. By tertiles, the levels fell from initial averages of 298, 250, and 204 mg per 100 ml to 252, 225, and 189 mg per 100 ml, respectively. The decreases in each tertile were statistically significant beyond the 1 per cent level. Control weights averaged 154.3 pounds; after six months on the diet, they averaged 153.0 pounds. In subjects with coronary heart disease, the cholesterol levels fell similarly to others with no clinical evidence of coronary heart disease and with the same benchmark levels.

This study demonstrates that the serum cholesterol level of a group of subjects can be significantly lowered (although not necessarily optimally) by adherence to a diet containing 9 to 11 per cent of total calories as polyunsaturated fatty acids with the saturated fatty acid component comprising 7 to 8 per cent of total calories.

Of 86 men placed on this diet originally, 79 (92 per cent) continued for at least six months, indicating a wide acceptance among our group of motivated subjects. We conclude that: (1) The prudent diet (which is described in detail) is practical and can be followed by the vast majority of sufficiently motivated subjects for at least six months, and (2) that this diet significantly lowers the average blood cholesterol level over a period of six months.

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Its members are: Director, Bureau of Preventable Diseases, Dr. Morris Greenberg; Director, Bureau of Laboratories, Dr. Gustav Steffen; Director, Diagnostic Center, Dr. Anthony Bianco; Director, Public Health Research Institute, Dr. George Hirst; Director, Bureau of Adult Hygiene, Dr. Abraham Oppenheim; and Director, Bureau of Records, Mr. Carl L. Erhardt.

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Diet Therapy

Practical Dietary Management of Patients with the Celiac Syndrome

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THE ABNORMALITIES in the celiac syndrome are numerous and complex. Although much progress has been made in the past few years, additional investigations must be pursued before there is a more complete understanding of basic hereditary and metabolic abnormalities. Various nomenclatures are used to define the celiac syndrome as a symptom complex. However, the basic clinical manifestations remain the same. The nomenclature in this discussion includes only the two most important conditions presenting the celiac syndrome, namely *idiopathic celiac disease* and *cystic fibrosis of the pancreas*. The emphasis will be on practical guides, as used in Babies Hospital, which may be helpful to the clinician in meeting the dietary requirements of the patient. Names of proprietary foods and vitamins are listed because their known ingredients have been demonstrated to be of clinical benefit. This does not imply that other products containing comparable ingredients could not be substituted.

To clarify any misunderstanding, in the discussion which follows, the term *starch* is used in a broad implication and designates a variety of complex carbohydrate foods and various grains. This should be kept in mind whenever the term appears and when interpreting the basic principles of the diets outlined. It is realized that in many cases of idiopathic celiac disease the gluten portion is at fault and not the starch fraction.

IDIOPATHIC CELIAC DISEASE

Classic Celiac Disease presents a marked intolerance to both dietary starch and fat, with steatorrhea and usually an excess of starch in the stools. The critical period may appear from six months to ten years of age, but usually appears during the second year. In the mild phase, sometimes termed *starch intolerance*, the outstanding clinical manifestation is intolerance to some types of dietary starch but a moderate amount of selected fats and oils are tolerated. Allergic manifestations are not uncommon. However, food intolerance may occur without an allergic basis.

As the patient improves, there is the gradual return to a normal diet with the nutritional requirements the same as for the well child. There may be a few individual restrictions, but for all practical purposes a regular diet is attained.

CYSTIC FIBROSIS OF THE PANCREAS

In this congenital, hereditary disease 80 to 85 per cent of patients with pancreatic deficiency require a large amount of food, especially of protein, to compensate for fecal loss. They have a chronic intolerance to fat. The use of selected starch varies in quantity according to the age and general condition of the patient. The only permanent restriction is the fat content of the diet. Pancreatin is to be used

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for all patients with pancreatic deficiency. However, for the remaining patients with normal pancreatic function, there are no dietary restrictions. It is important to emphasize increased salt intake in warm or hot weather to compensate for excessive loss of sweat chloride and sodium with resulting dehydration and vasomotor collapse.

TABLE I. BASIC PRINCIPLES OF DIET IN THE CELIAC SYNDROME

	Celiac disease	Fibrocystic disease
Vitamins: Aqueous multivitamin preparation	About 20 drops (1.2 ml), or 2 capsules daily; (vitamin A to be between 10,000 to 20,000 units); in mild phase about 10 drops or 1 capsule may be adequate	About 20 drops or 2 capsules daily; (vitamin A to be between 10,000 to 20,000 units)
B complex* (Lederplex)	2 to 4 teaspoonfuls daily, or 2 to 4 capsules	Same, depending on formula
Calories	Acute phase: 120 to 200/kg body weight; an excess to compensate for past and present fecal loss and failures in absorption; formula calories may cover the entire caloric needs in crisis, and be two-thirds of the total as the patient improves; *a very ill child requires frequent feedings*, every 2 to 3 hours day and night; soon after the acute phase of the disease, even for the very young, solids are added After acute phase: About 20 per cent above normal calculated requirements; gradual return to normal requirements	All ages and conditions: 50 to 75 per cent above normal requirements Newborn period: 150 to 200/kg body weight; the same as the celiac patient in the acute phase Older child: 120 to 150/kg body weight; between meal feedings are needed, especially for the older child, to prevent fatigue and excessive hunger; it may be necessary to use six small meals for some individuals
Protein	Acute phase: 6 to 8 g/kg body weight daily; we have had good results using Probana† NOTE: Do not feed only protein foods; an abnormally high blood level of nonprotein nitrogen may occur After acute phase: Gradual return to normal calculated requirements; allergies are not uncommon; therefore the use of fish, particularly shellfish, should be restricted	All ages and phases: 6 to 8 g/kg body weight daily; an excess is required at all times to compensate for the great loss; additional amino acids are required as part of the protein for infants under one year; this can be achieved by using Probana† formula
Simple carbohydrates and starches	Planned to make up the remaining calories Acute phase: Use only formula initially, then increase the use of well tolerated, relatively simple carbohydrates; included are glucose, fruit juice, *ripe* banana, cooked or canned fruit in syrup (the more complex carbohydrates in this limited amount are tolerated), and cooked vegetables; prunes and prune juice are usually not well tolerated; avoid the use of starch; patients originally having the severe phase should not have starch liberalized for six months to one year after the last severe bout of diarrhea	Planned to make up the remaining calories Acute phase and under one year of age: As outlined under the acute phase of celiac disease Over one year of age if not acutely ill: Gradually introduce starch; the selection is to be governed by the ability to chew as well as the socio-environmental needs; the quantity is limited to assure an adequate intake of other important foods. This will vary with the age and appetite of the individual child; in this phase there is no intolerance to starch

*,† See footnotes, page 466.

TABLE I (*Continued*)

	Celiac disease	Fibrocystic disease
Simple carbohydrates and starches (*Cont'd.*)	Mild phase (starch intolerance): After about 14 months of age, more liberal use of cane sugar is tolerated; *gradual* introduction of selected starch follows; there may be an intolerance to gluten in which case wheat, rye, oats, and barley would be avoided; the effect of new foods should not be judged until they have been given one week *or longer*, since the intolerance may not be manifested immediately; during an infection and in very hot weather, relapses may occur, requiring a return to a more restricted diet for a week or more; some restrictions may be required for years, as influenced by the clinical picture of the child and laboratory data; gluten-containing foods are poorly tolerated; there is individual variation, and they should be introduced on a trial and error basis	NOTE: The quantity of foods should never be restricted by the adult's attitudes, but foods should be offered to the point of satiety of the patient
Fat	Acute phase: Steatorrhea is present; the fat content of the diet is to be low; avoid visible fats and restrict to fat in the formula and a few selected foods; skim and whole milks, raw or pasteurized, and evaporated milks are not tolerated; however, protein milk, boiled skim and some types of dried skim milks are taken well; the fat in egg yolk and lean meat is not considered excessive and should be included in the diet Mild phase (starch intolerance): The tolerance varies; utilization is generally increased if the gluten content of the diet is initially kept low and the added fats are unsaturated (corn, cottonseed, soybean, sesame, and olive oils); progression to other fats, and a more liberal use of all fats and oils is made with clinical improvement of the patient	The fat content of the diet is to be low; limited absorption and tolerance is present; restrict intake to fats contained in the natural foods which help to provide the optimal dietary essentials as well as aid in planning a varied and practical menu; *if the patient is doing well and* is near adolescence, limited amounts of creamy peanut butter, mild cheese, lean ham, and crisp bacon (drained) can be used; homogenized milk may be used when away from home, as in school; some older patients may tolerate homogenized milk instead of skim milk Patients with normal duodenal enzymes: Fats are not restricted unless increased stools and digestive disturbances are produced
Roughage	Acute phase: Excessive roughage is not tolerated; the residue of pureed foods is not excessive Mild phase: Pureed foods are used for a longer period than for the well child. It is not uncommon to find undigested foods and colored foods excreted; do not restrict the offending foods; raw vegetables and additional raw fruits are added when there has been a marked clinical improvement—about three to four years of age	Acute phase: Same as in celiac disease. Over two years of age: There is no intolerance; the use is governed by the ability to chew

(*Continued on page 466*)

TABLE I (*Continued*)

	Celiac disease	Fibrocystic disease
Salt	Normal intake	All age groups: Increased intake, up to 6 g daily, in warm or hot weather; salt may be added to food or beverage or given as NaCl tablets *after* meals
Iron‡	When indicated use 10 mg of elemental iron/kg body weight; give with food other than milk	Usually not indicated
Vitamin E	Not required	The use is indicated for some patients, but further investigation is necessary
Vitamin K‡	Given when evidence of bleeding exists (prolonged prothrombin time); preparation—Hykinone® 5 mg administered intramuscularly until bleeding subsides	Same
Pancreatin *Powder:* 1 teaspoonful (2.0 gm) *Tablets:* 325 mg each *Granules:* 1/4 to 1/2 teaspoonful	Not required	Required for all patients with pancreatic deficiency; dosage varies with age and the number of meals scheduled *Powder:* 1/2 to 1 g (1/2 teaspoonful) in each bottle or 1 to 2 g (1 teaspoonful) with each meal on a three-meal-a-day plan *Tablets:* 1 to 3 tablets with each meal; older patients may require only 1 to 2 tablets as determined by the fat in the stools, on a trial and error basis *Granules:* 1/4 to 1/2 teaspoonful with each meal on a three-meal-a-day plan

* B complex: This should provide the estimated daily requirement of several B complex vitamins. The following is adequate: thiamine 1 to 2 mg, pyridoxine 0.5 to 1.0 mg, riboflavin 1 to 2 mg, pantothenic acid 10 mg, niacin 5 mg, folic acid 5 mg. Many proprietary preparations are satisfactory.

† Mead Johnson & Co., Evansville, Indiana. Contains: Protein Milk, protein hydrolysate, dextrose, and banana flakes, 4.4 calories/g dry weight.

‡ Dosage of iron and vitamin K suggested by the medical team at Babies Hospital to be used when considered necessary by the prescribing physician.

Following these principles, the general dietary pattern used during the course of the disease may be summarized (Table I):

(1) Start with formula for a young infant.

(2) Add selected fruits, protein foods, vegetables, and simple carbohydrates.

(3) Add a greater variety of fruits, vegetables, and additional amounts of cane sugar.

(4) Gradually add the more complex carbohydrates and starches, and raw fruits and vegetables.

(5) Since individual tolerance is variant for celiac patients, foods normally used in the family meal plan are introduced by trial and error in the return to a normal diet. Use a high protein, high caloric, modified normal diet with some fat restrictions for patients with pancreatic deficiency. Celiac patients, as a rule, tolerate fat earlier and in larger quantities, but initially require more restricted quantities of selected starch than do patients with pancreatic deficiency.

For ease in prescribing diets, a nomenclature has been established at Babies Hospital. The suggested type diets are guides only and are *always* modified to meet individual needs. The dietary outlines cannot be rigidly classified to be used at given ages for patients presenting the celiac syndrome.

GUIDE IN DETERMINING CALORIC REQUIREMENTS OF FORMULA

*Probana:** May supply the entire caloric requirement or be $^2/_3$ or $^3/_4$ of the total amount. The ratio of Probana to water should not be more concentrated than 1:4 by volume.

Hi Pro† or *Protein Milk:*‡ May be indicated. This should supply $^2/_3$ to $^3/_4$ of the total formula calories with the remaining $^1/_3$ to $^1/_4$ dextrose (glucose) and/or banana flakes or powder.

Water for these formulas is added to make the total volume 24 to 32 oz, or as demanded by the infant's needs.

It should be noted that Probana may be indicated for use in patients near or in celiac crisis or for all fibrocystic patients under one year of age. If not used previously, its unpleasant taste is not well accepted by patients over one year of age. One may then use a formula which is high protein and low fat, i.e., Hi Pro, or boiled skim milk for celiac patients, or double strength dried skim milk, flavored if desired, for patients with pancreatic deficiency.

GUIDE IN DETERMINING USE OF ADDITIONAL FOODS

Two to Four Months of Age {Table II}

In addition to the formula, the following foods may be offered in increasing amounts as governed by the appetite: Mashed *ripe* banana, banana flakes, and/or applesauce, scraped raw apple; finely ground or strained canned beef or liver; uncreamed skim milk cottage cheese, which may be served with jelly or honey; pureed string beans, carrots, and squash; egg yolk, increasing to whole egg; gelatin desserts; strained orange, grapefruit, apple, or tomato juice. When all these foods have been introduced, the nomenclature, is *strict to starch, high protein, low fat diet.*

Approximately Four to Fourteen Months of Age {Table II}

A formula is used. The diet is liberalized to include other lean meats, fruits, vegetables and miscellaneous items. Prunes and prune juice may not be tolerated by celiac patients. Occasionally peaches and spinach are not well tolerated in the beginning. If so, add them after the other foods have been given. The nomenclature for this diet is *no starch, high protein low fat diet.*

Allow only the following foods: Read labels carefully. Additional foods may be added at the discretion of the physician.

Pureed Vegetables (cooked, fresh, frozen, or canned)
- Asparagus
- Beets
- Carrots
- Peas
- Spinach
- String beans
- Squash
- Swiss chard
- Tomato juice

Fruits

Raw:
- Apple, peeled, thin sliced, or scraped
- Banana, ripe
- Pear (see apple)

Cooked or canned (Pureed):
- Applesauce
- Apricots
- Cranberries
- Peaches
- Pears
- Pineapple

Fruit juices (Strained):
- Apple
- Apricot
- Cranberry
- Grape
- Grapefruit
- Lemon
- Lime
- Orange
- Pear
- Pineapple
- Tangerine

Lean Meat, etc.
- Beef
- Chicken
- Heart
- Kidney
- Lamb
- Liver
- Turkey
- Veal

Fish (with caution for celiacs)
- Fresh (boneless)
- Low fat canned salmon, tuna

Miscellaneous
- Formula
- Uncreamed skim milk, cottage cheese
- Eggs—not in excess of three daily
- Extracts
- Fruit ices
- Fruit whips made with egg whites
- Gelatin desserts
- Hard candies, gum drops (when feasible)
- Honey
- Jelly, clear
- Marshmallows or sauce
- Meringues
- Rennet desserts
- Salt, other seasonings
- Sugar, syrups—all kinds

* Mead Johnson & Co. See note on page 466.

† Jackson-Mitchell Pharmaceuticals, Inc. A dried high protein, partially skimmed milk from which part of the lactose has been removed, 4.4 calories/gm dry weight.

‡ Mead Johnson & Co., Evansville, Indiana. A dried high protein mixture made from whole milk curd and lactic acid skim milk, 4.9 calories/g dry weight.

Approximately Fourteen Months to Two Years of Age {Table II}

The formula may be changed to powdered skim milk (double strength) for patients with cystic fibrosis and boiled skim or homogenized milk for those classified as having celiac disease. Buttermilk made from skim milk may be used. The basic diet is supplemented with restricted and selected starch. Unless gluten is restricted one may use zwieback, melba toast, progressing to one-half slice of crisp toast. Because of a generally precarious appetite, most patients should not use more than one serving of these foods three times a day. Progressively, puffed corn or rice cereals, arrowroot crackers, or social tea biscuits are added. The nomenclature is *low starch, high protein, low fat diet.*

Allow only the following foods: Read labels carefully. Additional foods may be added at the discretion of the physician.

Vegetables (Cooked fresh, frozen or canned)

Asparagus
Beets
Broccoli *tops*
Carrots
Cauliflower *tops*
Peas
Spinach
String beans
Squash
Swiss chard
Tomato juice

Miscellaneous

Milk—skim or homogenized (as tolerated)
Uncreamed skim milk, cottage cheese
Cocoa
Eggs—not in excess of 3 daily
Extracts
Fruit ices
Fruit whips made with egg whites
Gelatin desserts
Hard candy, gum drops
Honey
Jelly, clear
Marshmallows or sauce
Meringues
Rennet desserts
Salt, other seasonings
Sugars, syrups—all kinds

Fruits

Raw:
- Apple, peeled, thin sliced
- Banana, ripe
- Pear (see apple)
- Seedless grapes

Cooked or canned:
- Applesauce
- Apricots
- Cranberries, strained
- Peaches
- Pears
- Pineapple, strained

Fruit juices:
- Apple
- Apricot
- Cranberry
- Grape
- Grapefruit
- Lemon
- Lime
- Orange
- Pear
- Pineapple
- Tangerine

Lean meat, etc.

Beef, Chicken, Heart, Lamb, Liver, Turkey, Veal

Fish (with caution for celiacs)
Fresh (boneless)
Shellfish
Low fat canned salmon, tuna

*Starches** (1 serving 3 times daily of any *one*)
Zwieback
Melba toast
Crisp toast, 1/2 slice
Puffed-type cereals, 2 to 3 tablespoons
Arrowroot crackers or social tea biscuits, 2 to 3

* Avoid wheat, rye, oats, or barley if a low gluten diet is used.

Approximately Two to Four Years of Age

Homogenized milk is usually tolerated by celiac patients, while those with pancreatic deficiency generally require low fat milk. Celiac patients in the mild phase may tolerate moderate amounts of unsaturated fats (cottonseed, corn, sesame, olive, and soybean oils). They would usually not be used for those with pancreatic deficiency. Progress to a *moderate starch, high protein, low fat (or moderately low fat) diet*, with restrictions of foods containing wheat, rye, oats, or barley for the celiac patient, when indicated. Additional suggested starches include:

Potato or rice
Cereals, puffed-type initially: add cooked ones later
Angel food or sponge cakes, lady fingers
Puddings, cream soups
Macaroni, spaghetti, noodles, plain bread, saltines, soda crackers, etc.

The quantity of starch is governed by the appetite, but should never replace basic foods necessary to maintain good nutrition.

It should be noted that legumes, as dried lentils, baked beans, lima beans, and others, frequently cause discomfort and should be avoided or used with caution when progressing to a more liberal diet.

Approximately Three to Six Years of Age

Additional foods are introduced by trial and error, including raw vegetables, raw fruits previously restricted, avoiding those foods causing discomfort. When there is clinical improvement in the celiac patient, other fats may be used in addition to those already allowed. These include: creamy peanut butter, vegetable shortening and finally ice cream and butter. *These fats would not generally be intro-*

TABLE II

Meal	Strict no starch high protein low fat	No starch high protein low fat	Low starch high protein low fat
Breakfast	Citrus or apple juice (strained) Ripe banana, banana flakes or applesauce Eggs—no added fat Uncreamed skim milk cottage cheese or meat, if desired Formula	Citrus juice (strained) Ripe banana or other fruit from list Eggs—no added fat Uncreamed skim milk cottage cheese or meat, if desired Formula	Citrus juice (strained) Ripe banana or other fruit from list Eggs—no added fat Uncreamed skim milk cottage cheese or meat, if desired Starch—1 serving Milk or formula
Dinner and supper	Finely ground or strained canned beef or liver (2 or more tablespoons) Cheese from list may be used for *one* meal Pureed string beans, carrots or squash (1 or more tablespoons) Dessert: gelatin desserts, applesauce, scraped raw apple, ripe banana, or banana flakes Formula	Finely ground or strained canned lean meat, poultry (or fish); *cut off all visible fat;* broil, boil or roast Cheese from list or egg may be used for *one* meal Pureed cooked vegetable Dessert: pureed fruit should be used for *one* meal Formula	Finely chopped or ground lean meat, poultry or fish; *cut off all visible fat;* broil, boil or roast Cheese from list or egg may be used for *one* meal Cooked, fresh, frozen, or canned vegetable (pureed or chopped, dependent on age) Dessert: fruit should be used for *one* meal Starch—1 serving Milk or formula
Evening	Formula	Formula	Milk or formula
Between meal feedings	Dependent on appetite	Dependent on appetite	Any foods on diet list, except starch in excess of allowance, if they do not interfere with the appetite at mealtime NOTE: These feedings should always be planned for those with cystic fibrosis

duced into the diet of the patient with pancreatic deficiency.

Approximately Over Six Years of Age

This represents a transitional stage. The quantity of food eaten should be unrestricted and the daily intake should include foods essential to optimal nutrition.

Idiopathic celiac disease: In patients with this disease the caloric and nutritional requirements are essentially the same as for a well child. Foods from the family meal plan are introduced by trial and error. Individual variations will occur. Administration of a vitamin B complex preparation should be continued. Intake of aqueous multivitamins is the same as for the well child.

Older patients with fibrocystic disease and pancreatic deficiency: The protein, caloric, and other nutritional requirements remain increased with the intake of fat moderately restricted. Although the amount of fat tolerated varies with the individual patient, it should always be used in moderation, as suggested in the *high protein, high caloric, moderately low fat diet* plan. Some patients must continue with rigid fat restrictions to prevent steatorrhea and discomfort. There are no other dietary restrictions. The use of frequent feedings is generally indicated. Aqueous multivitamins, vitamin B complex, and pancreatin are to be continued.

A suggested dietary plan for the older patient with fibrocystic disease follows (Table III):

HIGH PROTEIN, HIGH CALORIC, MODERATELY LOW FAT DIET (For older patients with fibrocystic disease)

Breakfast

Citrus juice
Fruit, if desired
Cereal, whole grain or enriched, with milk and sugar
Eggs
Bread, toast or plain roll, whole grain or enriched
Jelly, jam
Butter or margarine—1 teaspoonful (if tolerated)
Milk: skim (homogenized, if tolerated)
Pancreatin—1 teaspoonful

Dinner and Supper

Lean meat, poultry or fish; *Cut off all visible fat;* broil, boil, or roast; cottage cheese, mild cheese, or egg may be used for *one* meal
Potato or substitute
Vegetables, cooked and raw
Bread, whole grain or enriched
Jelly, jam
Butter or margarine—1 teaspoonful (if tolerated)
Dessert: Fruit, gelatin desserts, rennet desserts, custard, fruit ices, low fat desserts, cake or cookies (within limits of the prescribed diet) or puddings
Milk: skim (homogenized, if tolerated)
Pancreatin—1 teaspoonful

Between Meal Feedings

Any food on diet list; these feedings should always be included in the meal plan

TABLE III. DIET FOR OLDER PATIENTS WITH FIBROCYSTIC DISEASE

	Foods allowed	Foods to avoid
Milk	Skim—liquid or dry, low fat buttermilk, and yogurt (homogenized milk, if tolerated)	Homogenized milk (unless tolerated) Cream—sweet or sour
Eggs	1 to 3 daily, boiled, poached, scrambled in skim milk unless fat is allowed	Fried or with added fat *unless* fat is allowed
Meat	Lean beef, chicken, lamb, liver, kidney, heart, turkey, veal Fish: fresh, shellfish, low fat canned salmon or tuna NOTE: May use *limited* amounts of *all beef* frankfurters (boiled), lean ham and bacon (well drained)	Fat meats such as pork, sausage, duck, goose, luncheon meats, corned beef, pastrami Fat fish such as mackerel, sardines, swordfish, herring, or fish canned in oil or cream Smoked fish such as salmon
Cheese	Cottage cheese; mild varieties such as American or Swiss in moderation	All others
Vegetables	All cooked, frozen, canned, or raw, as tolerated	Those causing discomfort; these may include corn, strong flavored vegetables, and dried beans, peas, limas, or lentils
Potato	White or sweet (boiled, baked, or mashed)	Fried, potato chips
Fruits, juices	All fresh, frozen or canned, as tolerated	Avocado and those causing discomfort which may include prunes or prune juice
Breads, crackers, etc.	Whole grain or enriched breads, soft or hard rolls, bagels; soda crackers, saltines, matzoth, social tea biscuits, arrowroot crackers, popcorn (no oil) Thin vanilla cookies, Fig Newtons, ginger snaps, "slim" pretzels or graham crackers (in moderate amounts)	Hot breads, pastries, muffins, biscuits, "short" crackers or cakes, doughnuts
Cereals	All breakfast cereals except bran and shredded wheat Rice, noodles, macaroni, spaghetti, kasha (buckwheat groats), pastina, barley, grits	Bran, shredded wheat (unless tolerated)
Fats	Creamy peanut butter or drained bacon in limited amounts If tolerated, butter, fortified margarine, oil, salad dressing—not in excess of 1 teaspoonful at each meal	Butter, fortified margarine, oil, salad dressings (unless tolerated in limited amounts), all other fats, cream—sweet or sour

TABLE III (*Continued*)

	Foods allowed	Foods to avoid
Soups	Broth, bouillon—without fat; cream soups —without fat and made with milk allowed	Fatty soups, those made with added cream —sweet or sour; lentil and dried bean soups if they cause discomfort
Beverages	Cocoa, milk—skim or homogenized (as tolerated), fruit juices; weak tea (when indicated); *in moderation*—carbonated beverages	Chocolate, coffee, unlimited amounts of carbonated beverages, milk shakes, malted milk powder (unless whole milk is tolerated)
Sweets	Sugar, jelly, jam, honey, syrups, molasses, mints, hard candies, gum drops, marshmallows, meringues	Chocolate candies or those made with nuts, added fats or oils, creamed
Desserts	Gelatin desserts, custard, rennet desserts, puddings as cornstarch or tapioca, fruit ices, fruits, low fat desserts, fruit whips made with egg whites, angel food or sponge cakes NOTE: Plain ice cream (inexpensive type) may be used once a week; plain cake with low fat frosting may be used occasionally	Pie crust, rich pastries or desserts, frostings made with fat, rich ice creams or those containing nuts
Miscellaneous	Cocoa, Bosco, and other similar products, raisins, dates, salt and other seasonings, extracts, pancakes or waffles without fat	Fried foods, scrapple, gravies and sauces made with fat, nuts, pizza pie, ravioli, mixed dishes made with fat, olives, Fritos, and other similar foods
Salt	Use liberally in warm or hot weather	—
Vitamins	Aqueous multivitamins; B complex (Lederplex)	—
Pancreatin	Three times daily at mealtime	

THE ROLE OF GLUTEN IN IDIOPATHIC CELIAC DISEASE

Celiac disease is thought to be a basic constitutional defect. The disease is familial in nature and heredity is an important factor. Gluten does not cause the symptoms, but may be one of the trigger mechanisms. Not every patient with normal pancreatic function and chronic steatorrhea responds to a *low gluten diet** in which wheat, rye, oats, and barley are omitted. There is also a varying threshold for gluten tolerance among children.[39] Some respond to a diet containing selected wheat products, i.e., melba toast, zwieback, angel food, or sponge cakes, if used in limited amounts. However, they do not tolerate other wheat, rye, oat, or barley foods, such as breads, cereals, noodles, and other pasta, until they are near adolescence or beyond. Other children show marked improvement on a low gluten diet in which all wheat, rye, oat, and barley products are omitted, as feasible. *For these children restrictions must be as complete as possible*, otherwise there is no effective response. The length of time restrictions are necessary has not been definitely established, but it is thought to be until active growth has ceased.

The fat tolerance in celiac disease is in direct proportion to the tolerance of gluten. The more highly unsaturated fats (corn, cottonseed, soybean, sesame, and olive oils) are used in limited amounts in the early stage of the *mild phase*. Their quantity is gradually increased with improved appetite and clinical status. Following this, the polysaturated fats may be introduced. Hard fats of meat and bacon are usually poorly tolerated, as are foods prepared with excess fat.

It is important to include a suggested meal

* The term *low gluten diet* has been used as opposed to *gluten-free diet* because it is impossible to eliminate all of the offending agent. As stated in the text, there is evidence[39] that a small amount of gluten is tolerated by almost all patients.

TABLE IV. LOW GLUTEN DIET FOR PATIENTS WITH CELIAC DISEASE

Circle foods allowed. Read labels before you use a product

NOTE: The foods allowed will vary with the clinical course of the individual and should be adapted

	Foods allowed	Foods to avoid
Beverages	Milk—plain or flavored with chocolate, cocoa, or products as Bosco, Ovaltine, Cocoa Marsh Buttermilk Fruit juices Soda or other carbonated beverages *on special occasions* only, in limited amounts	Malted milk, Cocomalt, Hemo, Postum, Nestle's Food and the like
Meat or substitute	All meat, trimmed of fat, except those listed under foods to avoid Eggs, cheese Poultry Fish: fresh, frozen, canned, shellfish, except those listed under foods to avoid Creamy peanut butter (when tolerated)	Fat meats such as pork, ham, sausage, luncheon meats, corned beef, duck, goose, frankfurters Fat fish such as: mackerel, swordfish, sardines, herring, or fish canned in heavy oil or cream Smoked fish such as salmon Meat prepared with bread, crackers, or flour (such as meat loaf, stew) Commercially prepared meat products where wheat may be used as a filler (as frankfurters, sausage, luncheon meats, meat loaf, scrapple)
Fats and oils	Oils: cottonseed, corn, soybean, sesame, olive Olives French dressing, true mayonnaise, Durkee's or other dressings made with cornstarch Creamy peanut butter, when tolerated *Later additions:* margarine, vegetable shortening (Crisco, Spry, etc.), yogurt, avocado, peanut oil, cream, ice cream, cream cheese, butter	Suet, bacon, lard Salad dressings if flour is used for thickening (Miracle Whip, etc.) Coconut or oil Excessive use of chocolate Nuts *Until added at a later time:* margarine, vegetable shortening (Crisco, Spry, etc.), yogurt, avocado, peanut oil, cream, ice cream, cream cheese, butter
Fruits and juices	All cooked or canned fruits and juices Frozen or fresh fruits (as tolerated)—may have to avoid seeds and skins initially; avoid those causing discomfort	Prunes, plums, or their juices, unless tolerated
Vegetables	All cooked, frozen, canned as tolerated, if prepared without wheat, rye, oat, or barley products Raw, as tolerated (avoid initially if diet has been restrictive)	Those causing discomfort; these may include corn, strong flavored vegetables, and dried beans, peas, limas, lentils, etc., pickles All prepared with wheat, rye, oat or barley products
Soups	Broth, bouillon—without fat, cream, or other soups made without wheat, rye, oat, or barley products	All soups containing wheat, rye, oat, or barley products
Sweets and sugars	All sugars, syrups, honey, molasses, jelly, jam, marmalade, candies if not made with wheat, rye, oat, or barley products, or nuts Marshmallows or sauce Meringues	All candies containing wheat, rye, oat, or barley products, or nuts Avoid excessive use of candies with high fat content (as most candy bars)

TABLE IV (*Continued*)

	Foods allowed		Foods to avoid	
Starches Cereals	*Prepared* Corn Soya Cornflakes Cheetos Infant type—rice, corn Puffed Rice Pure rice cereals Rice Chex Rice Honeys Rice Krispies Sugar Cornfetti Sugar Corn Pops or Frosted Flakes Sugar Rice Krinkles	*Cooked* Buckwheat groats (Kasha) Cornmeal Cream of Rice Hominy or Grits Rice	All-Bran Barley, pearled Beemex Bran Flakes Cheerios Crackles Cream of Wheat Farina Grapenuts and Flakes H-O Oats Infant type—mixed, oat, barley Jets Kellogg's K Kix Krumbles Maltex Maypo Mello-wheat Muffets New Oats	Oatmeal Quaker Oats Pep Pettijohn Protein Plus Puffed Wheat Ralston—Regular, Instant, Shredded Shredded Wheat Sugar Crisp Sugar Smacks Trix Wheat Chex Wheat Flakes Wheat Honeys Wheat germ Wheat Puffs Wheaties Wheatsworth Wheatena Whole Bran
Potato	White or sweet, Yams		—	
Breads, Flours	Corn pone, breads, muffins, and other baked goods prepared with these *suggested flours:* potato, soybean, rice, cornmeal, cornstarch, buckwheat, lima bean (gravies, soups, and sauces may be thickened with cornstarch)		All bread, toast, biscuits, rolls, bagels, muffins, cornbread and other baked products prepared with gluten, wheat, rye, oats, or barley Gravies, sauces, dumplings and other similar products prepared with gluten, wheat, rye, oats, or barley	
Crackers, etc.	Popcorn, Fritos, potato chips, Cracker Jacks, and special products not containing gluten, wheat, rye, oats, or barley NOTE: These foods are added with progression of diet		All crackers, cookies, rusks, pretzels, matzoth, zwieback, rye wafers, Ry-Krisp, and others prepared with gluten, wheat, rye, oats, or barley	
Desserts—Miscellaneous	Puddings as cornstarch, rice, tapioca Custard, fruit ices, ice creams without nuts, sherbets, rennet desserts, gelatin desserts, and fruit whips made with egg whites Special cakes or cookies made with flours allowed, and not containing gluten, wheat, rye, oats, or barley		Pies, cookies, cakes, doughnuts, ice creams with nuts, bisques, ice cream cones, prepared mixes unless label proves they are made without gluten, wheat, rye, oats, or barley	
Miscellaneous	Griddle cakes, pancakes or waffles prepared without gluten, wheat, rye, oats, or barley (use in latter part of dietary progression) Indian pudding (cornmeal) Seasonings as desired. Malt extract or malt flavoring in prepared foods and beverages Soy sauce		Griddle cakes, pancakes, pancake mixes, waffles, macaroni, spaghetti, noodles, pastina or other pasta made with wheat, rye, oats, barley, or gluten	
Vitamins	Aqueous multivitamins; B complex (Lederplex)		—	

plan when giving instructions to patients. Frequently parents cannot formulate such a plan when given only a list of foods to allow and avoid. Also, parents should be alerted to read labels on all prepared foods carefully and to avoid those not proving to be made without gluten, wheat, rye, oats, or barley.

Low gluten diet for celiac patients: When necessary to use a low gluten diet, the following outline can be adapted to the clinical status and age of the individual patient. *Modifications should be clearly defined by some means,* such as circling the items allowed (Table IV). A suggested meal plan follows:

LOW GLUTEN DIET FOR CELIAC PATIENTS*
(Omit wheat, rye, oat, and barley products)

Breakfast

Citrus fruit or juice
Starch, —— serving(s)
Eggs
Milk, homogenized
Fats and oils (specify kind and amount)
Jelly, sugars, etc.

Dinner and Supper

Lean meat, poultry, fish; *cut off all visible fat*; broil, boil, or roast; cheese, egg (or creamy peanut butter, if tolerated) may be served for *one* meal
Potato or starch substitute, —— serving(s)
Vegetables, cooked or raw
Fats and oils (specify kind and amount)
Jelly, sugars, etc.
Dessert: Fruit should be used for one meal
Milk, homogenized
(Special soups without gluten, as desired)

Between Meal Feedings

Any food on diet list, if appetite is good

* Grateful acknowledgment is given to Jane Andrews, Clinic Dietitian, Vanderbilt Clinic, Presbyterian Hospital, New York, for her contributions in the preparation of this diet.

GENERAL DISCUSSION

The clinical status of the individual child, pathologic manifestations, length of disease, and the individual appetite govern the dietary regimen to be followed. Celiac patients who present only mild symptoms, without malnutrition, steatorrhea, or severe diarrhea, do not have to be started on the first, more restrictive stage. An acute respiratory infection generally lowers the patient's tolerance to foods.

If behavior problems are present, they should be simultaneously corrected. These children are emotionally unstable and react strongly to environmental stimuli. Therefore, patience and understanding of the emotional difficulties are required of the team interviewing and treating the patient. This factor has tremendous influence on the effectiveness of the treatment.

It is equally important to consider the socioeconomic status of the family because celiac disease is a long-term illness. Practical suggestions for planning menus and preparing food should be included when instructing patients. In planning to meet some of the social needs of the older patient with pancreatic deficiency, one may occasionally suggest dietary indiscretions without producing harmful effects. An example may be the use of homogenized milk when away from home, as in school. This may have a tremendous influence on the patient's psyche.

SUMMARY

The two most important conditions presenting the celiac syndrome include *idiopathic celiac disease* and *cystic fibrosis of the pancreas*. The emphasis in this discussion is on practical guides which may be helpful to the clinician in meeting the dietary requirements of the patient with this syndrome.

The basic principles of dietary management as used in Babies Hospital are described. The general discussion includes dietary patterns followed during the course of the disease and guides in the use of diets according to age, clinical status and pathologic conditions. Suggested meal plans and food guides are included for the various age groups. A low gluten diet is outlined in detail.

The references selected are a few which are useful in understanding the celiac syndrome as a symptom complex and its treatment. The suggested treatment presented is a composite of many years of observation and research by our medical team and that of other groups.

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Nutrition News

National Vitamin Foundation States Research Interests, Invites Applications

The National Vitamin Foundation wishes to bring to the attention of those engaged in clinical and basic research in the fields of nutrition, biochemistry, and metabolism, its current major interest in the support of research, preferably on man, most likely to advance our understanding of the diverse metabolic functions of vitamins. It believes that procedures and facilities now are available to permit the profitable exploration of nutritional and metabolic aspects of mental disease, and the effects of neuropharmacologic agents on nutritional status. It is also of the opinion that, in many instances, dermatologic reactions to ingested drugs may have a common denominator in susceptible persons, and that the basic fault may be one of nutrition and metabolism. The Foundation wishes to encourage research on vitamin nutrition and metabolism in the areas of (1) mental health and (2) skin eruptions related to drug ingestion and allergies, and, therefore, invites applications for grants-in-aid of such research from interested and qualified investigators.

This statement of the current major research interests of the Foundation should not be interpreted as eliminating consideration by the Foundation of applications for grants-in-aid of research on other aspects of vitamin metabolism and nutrition. Research projects in the two areas cited, however, will receive preferential consideration, other things being equal.

Application forms for grants-in-aid can be obtained from the offices of the Foundation at 149 East 78th Street, New York 21, N. Y. For grants to be effective July 1, applications should be mailed to the Foundation before the preceding March 1; for January grants, applications should be in the possession of the Foundation by September 15.

Nutrition Education Programs

The National Vitamin Foundation, in active cooperation with the Commissions on Nutrition of the Pennsylvania State Medical Society and the Philadelphia County Medical Society, is developing nutrition education programs for the practicing physician. It is planned to present at the monthly meetings of the various county medical societies a discussion of the basic and practical aspects of nutrition as they relate to disease. The following programs have been scheduled:

Date	*Speaker*	*Subject*	*Society and location*
September 15	HERBERT POLLACK, M.D.	Complications of Diabetes–Nutritional Aspects of the Disease and Oral Hypoglycemic Agents*	Allegheny County Medical Society, Pittsburgh, Pa.
September 21	PAUL GYÖRGY, M.D.	Nutrition in Liver Disease	Montour County Medical Society and Geisinger Memorial Hospital Staff, Danville, Pa.
September 26	ROBERT G. RAVDIN, M.D.	Nutritional Problems Associated with Surgery	Pittsburgh Hospital Scientific Day Project, Pittsburgh, Pa.
October 14	JAMES H. SHAW, PH.D.	The Role of Nutrition in the Practice of Dentistry	Philadelphia County Dental Society, Philadelphia, Pa.
October 21	PAUL GYÖRGY, M.D. W. H. SEBRELL, JR., M.D. ROBERT G. RAVDIN, M.D. THOMAS MACHELLA, M.D.	Panel discussion: Nutritional and Metabolic Considerations in Disease	Philadelphia General Hospital (Interns and Residents of Philadelphia Area), Philadelphia, Pa.
November 3	ELAINE P. RALLI, M.D.	Diabetes Mellitus	Dauphin County Medical Society, Harrisburg, Pa.
November 18	PAUL GYÖRGY, M.D. PAUL SHALLENBERGER, M.D.	Hepatobiliary Disease	Bradford County Medical Society, Sayre, Pa.

* Other topics of the symposium to be considered by various speakers from the Pittsburgh area are: Cardiovascular Complications; Ophthalmologic Complications; Pre- and Postoperative Management of the Diabetic Patient; Juvenile Diabetes.

Reviews of Recent Books

Cholesterol, by David Kritchevsky. John Wiley & Sons, New York, 1958, pp. 291, $9.75.

Concise and accurate reviews are an asset in any field of research but are almost essential in that of atherosclerosis and lipid metabolism where so much has been written. Cholesterol occupies a key position in the study of atherosclerosis and Dr. Kritchevsky's monograph will find many eager readers, particularly when it is known how packed with information it is.

The book is divided into seven chapters, dealing with the chemistry, biosynthesis, adsorption and transport, and metabolism of cholesterol, with cholesterol in disease, the blood cholesterol, and analytical technics. It is difficult to understand why a separate chapter has been devoted to the blood cholesterol, as the information it contains might more logically have been incorporated into the chapters dealing with cholesterol metabolism in health and disease. There are detailed appendices on the physical constants of cholesterol and many of its compounds and derivatives and on the cholesterol content of human and animal tissues and of foods.

The style is admirably concise, and the book is easy to read. One of its most valuable features is the list of references at the end of each chapter. A total of 2086 sources of information is listed. There are few typographical errors and the printing and binding are good.

Dr. Kritchevsky is to be congratulated on writing what for many interested in atherosclerosis and lipid metabolism will probably be the "book of the year" as well as an amazingly compact and permanently useful reference book.

G. WALKER

Food Habits and Nutrient Intakes in a Siamese Rice Village: Studies in Bang Chan, 1952–1954, by Hazel M. Hauck, Saovanee Sudsaneh, and Jane R. Hanks. Cornell Thailand Project, Interim Reports Series Number 4, Data Paper Number 29, Southeast Asia Program, Department of Far Eastern Studies, Cornell University, Ithaca, N. Y., May, 1958.

This study of food habits and nutrient intakes in a Siamese rice village was a part of the Cornell Thailand project, which is under the auspices of the Department of Sociology and Anthropology of Cornell University. The dietary studies were made during a period from September, 1952, through May, 1954.

The data can be divided into two general categories: (1) qualitative information obtained from observation of the field party, interviews with mothers, and records of food eaten for 24 hours by grade-school children and (2) quantitative information provided by a weighed dietary study of eleven families.

The sections of the report concerning the community, social factors affecting nutritional level, the Buddhist priesthood, and general descriptions are very interesting. The quantitative family studies are thoroughly documented.

Although reproductions of photographs are expensive, it is too bad a few pictures could not have been included. The sample of eleven families, or 3 per cent of the families in the area representing 69 persons or 4 per cent of the population, for four nonconsecutive days during the year may be questioned as not being sufficient to produce a pattern of food intake. However, the quantitative material was supplemented with other data to make it meaningful. The reviewer appreciates the amount of time and effort which the field workers expended to secure the information. The report is well organized and is interesting as well as informative.

MARTHA F. TRULSON

BOOKS RECEIVED

Books received for review by THE AMERICAN JOURNAL OF CLINICAL NUTRITION are acknowledged in this column. As far as practicable, those of special interest are selected, as space permits, for a more extensive review.

Annual Review of Medicine, Volume 10, 1959, edited by David A. Rytand, Annual Reviews, Inc., Palo Alto, Calif., 1959, pp. 448, $7.00.

Conférence Internationale sur L'Influence des Conditions de Vie et de Travail sur la Santé, L'Association Medicale Internationale pour L'Étude des Conditions de Vie et de la Sante, Vienna, pp. 607, $6.00.

Hypertension: The First Hahnemann Symposium on Hypertensive Disease, edited by John H. Moyer, W. B. Saunders Co., Philadelphia, 1959, pp. 790, $14.00.

A Textbook of Medicine, edited by Russell L. Cecil and Robert F. Loeb, W. B. Saunders Co., Philadelphia, 1959, pp. 1,665, $16.50.

The Year Book of Endocrinology (1958–59 Year Book Series), edited by Gilbert S. Gordon, Year Book Publishers, 1959, Chicago, pp. 384, $7.50.

Blood Groups, May 1959 issue of the British Medical Bulletin, Vol. 15, No. 2, The British Council, London, pp. 86, $3.25.

Low-Fat Diet: Reasons, Rules and Recipes, by Roy L. Swank and Aagot Grimsgaard, University of Oregon Books, Eugene, 1959, pp. 143, $2.50.

Abstracts of Current Literature

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AMINO ACIDS AND DISEASE

Cystine is one of the least soluble amino acids which in the genetically determined disease cystinuria leads to the formation of renal calculi composed almost entirely of this substance. Lysine, arginine, and ornithine are excreted in abnormal amounts in the urine of these patients. The reduced level of amino acid excretion in patients with renal calculus formation other than cystinuric patients requires confirmation.

The Urinary Excretion of Amino Acids in Calculus Patients. M. G. McGeowan. *Scandinav. J. Clin. & Lab. Invest.* 10 (Suppl. 31): 290, 1958.

It has been shown in vitro that amino acids increase the solubility of such sparingly soluble salts as calcium and magnesium phosphate, and this is particularly true at an alkaline reaction. This suggests that patients with stone might exhibit abnormalities of amino acid excretion. In this brief abstract it is reported that by use of paper chromatography and determination of the urinary amino nitrogen excretion, 42 per cent of patients with calculus were found to excrete reduced amounts of amino acid in the urine. Thirty-two per cent of patients have been found to excrete increased amounts of cystine.

S. O. WAIFE

Current investigations on various metabolic defects of a hereditary nature have disclosed many single enzyme deficiencies in tissues or serum which account for the clinical abnormalities. The metabolic block established by the inherited inability to form a specific enzyme may have both direct and secondary effects in cellular formation.

The Nature of the Defect in Tyrosine Metabolism in Alcaptonuria. B. N. La Du, V. G. Zannoni, L. Laster, and J. E. Seegmiller. *J. Biol. Chem.* 230: 251, 1958.

Alcaptonuria is a hereditary disorder in which tyrosine is oxidized to homogentisic acid which is excreted in the urine. Enzymes which participate in the catabolism of tyrosine were studied in a biopsy sample of liver from an alcaptonuric patient. The pathway of tyrosine oxidation in normal human liver homogenate was found to be similar to that in other mammalian species, namely, L-tyrosine → p-hydroxyphenylpyruvic acid → homogentisic acid → maleylacetoacetic acid → fumarylacetoacetic acid → acetoacetic acid and fumaric acid. Homogentisic acid oxidase was absent in the alcaptonuric liver homogenate. Since 2,5-dihydroxyphenylpyruvic acid and 2,5-dihydroxyphenyl-DL-alanine were not oxidized by human liver homogenates, they were not considered to be intermediates in the catabolic oxidation of tyrosine. It was suggested that the alcaptonuric defect may be due to a failure in the synthesis of active homogentisic acid oxidase.

M. K. HORWITT

While the effect of whole-protein feeding and withdrawal upon hormone production by various endocrine glands has been widely investigated, there is scant information concerning the influence of combinations of amino acids upon this function.

The Effect of Amino-Acids on Pituitary–Adrenal Activity. A. Goth, N. Nadasdi, and E. Stadler. *Ztschr. Vitamin- Hormon- u. Fermentforsch.* 9: 184, 1958.

Compensatory hypertrophy of the right adrenal after removal of the left can be increased by daily administration of methionine, leucine, or arginine. The ascorbic acid depletion in the right adrenal after the removal of the left can be increased by giving leucine; this effect was eliminated following hypophysectomy. In intact rats, leucine and methionine decrease the ascorbic acid content of the adrenals. Protein intake increases

the urinary output of corticoids in man 70 per cent of cases. Compensatory hypertrophy failed to develop on a protein-free diet. These findings, and the eosinopenic effect of amino acids, suggest that amino acids exert a stimulating effect on the hormone secretion of the pituitary. It may be supposed that the intake of food is one of the physiologic impulses governing hormone secretion. AUTHORS

Influence of Amino Acid Therapy in Chronic Idiopathic Pruritus Ani. A. J. Levine, O. F. Davis, B. Horwitz, N. Sloan, and C. Beck. *Gastroenterology* 35: 409, 1958.

Twelve amino acids were studied in ten patients each with pruritus ani. Moderate benefit was obtained with tryptophan, phenylalanine, leucine, and valine. No change or mild to moderately severe irritation was observed with arginine, histidine, lysine, tyrosine, cystine, methionine, threonine, and isoleucine.

In an extension of this study a commercially available ointment was used, containing a polyethylene glycol base, lactalbumin hydrolysate at a pH of 6.1, and the following amino acids: arginine, histidine, lysine, tyrosine, tryptophan, phenylalanine, cystine, methionine, serine, threonine, leucine, valine, and alanine. Two hundred twenty-six patients with chronic idiopathic pruritus ani were treated with this ointment. After ten days, 71 per cent were improved and 59 per cent completely healed. Of 33 patients treated with adrenal corticosteroid ointments, 73 per cent were improved and 30 per cent completely healed. In 70 patients exposed to placebo treatment, 12 per cent showed improvement of the lesion and 20 per cent reported relief of pain and itching. Topical anesthetics were observed to produce no measurable improvement of the lesions; worsening was observed in 11 of 21 cases.

The authors conclude that the results with the amino acid ointment are encouraging. They are, however, able to present no data indicating any pharmacologic effect of these agents. Their rationale for the use of the treatment is the fact that regenerative tissue requires large quantities of amino acids to synthesize new protein. The authors point out that in chronic pruritus ani, the alkalinity of the anal mucosa arises from slight alkalinity to a pH of 8 to 10. The increased alkalinity may contribute to excoriation. Their studies did not rule out the fact that the lactalbumin hydrolysate at a pH of 6.1 might have been the effective agent in their ointment and might have produced its effect solely by a pH change. J. B. HAMMOND

Hematologic Manifestation of Lysine Deficiency in Swine. J. W. Athens, G. E. Cartwright, and M. M. Wintrobe. *Proc. Soc. Exper. Biol. & Med.* 97: 909, 1958.

Twenty-three swine of the Chester-White breed were fed a synthetic diet deficient in lysine for periods up to 160 days. After 130 days, two animals received supplements of L-lysine; five others were given the lysine antagonist hexahomoserine. After four weeks of feeding the lysine-deficient diet, an anemia developed that increased as time went on. This anemia was normocytic and normochromatic, and accompanied by hypoalbuminemia and hypocupremia. There were no changes in the iron content of the plasma or in the cellular constituents of the bone marrow. The anemia responded readily to the treatment with lysine. The latter is thus essential for normal erythropoiesis of swine. M. SILBERBERG

On the Efficacy of Asparagine, Glutamine, Alpha-Aminobutyric Acid and 2-Pyrrolidinone in Preventing Chemically Induced Seizures in Mice. J. E. Hawkins, Jr. and L. H. Sarett. *Clin. chim. acta* 2: 481, 1957.

It has been known for some time that L-asparagine and L-glutamine can reverse defects in the metabolism of epileptogenic cerebral cortex *in vitro*. In clinical trials the two amides are known to exert some control over seizures and to improve the electroencephalogram. A need for an evaluation test prompted the authors' studies. Mice were protected against convulsions induced by β- methyl-β-ethylglutarimide (Megimide), but not against electroshock-induced convulsions, by large doses (400 mg/kg) of α-aminobutyric acid and its lactam 2-pyrrolidinone. In man 1 to 2 mM/kg of 2-pyrrolidinone orally produces no untoward effects except gastrointestinal irritation. Alpha-aminobutyric acid causes nausea and dizziness, which disappears during continued administration. There is rapid complete absorption. Asparagine and glutamine, on the other hand, were less effective. Clinical tests are in progress to determine efficacy of the two amides as antiepileptic agents. E. COHEN

Imbalance in amino acid feeding in experimental animals has provided valuable information for nutritionists. It is possible to produce anemia, fatty livers, and growth retardation singly or in combination, together with other signs of protein deficiency and nitrogen loss, by administration of an unbalanced amino acid composition. Alterations in the dietary amino acid formula can be made which will eliminate one or all of the manifestations.

Chemical Pathology of Acute Amino Acid Deficiencies: I. Morphologic Changes in Immature Rats Fed Threonine-, Methionine-, or Histidine-Devoid Diets. H. Sidransky and E. Farber. *A.M.A. Arch. Pathol.* 66: 119, 1958.

Male and female rats of the Sprague-Dawley strain one month old and weighing 80 gm. were fed a basal diet for one week. Subsequently the animals were kept on diets free of (1) threonine, (2) methionine, and (3) histidine. These diets were fed ad libitum, or forced feeding was carried out twice daily by means of a stomach tube, 0.9 gm. of diet per 10 gm. body weight being given. Forced feeding of the methionine-devoid diet for seven days caused periportal fatty change with deposition of excessive amounts of glycogen in the liver,

edema of the pancreas, and atrophy of salivary glands, gastric mucosa, and adrenal cortex. Animals fed ad libitum did not show marked changes, probably because they consumed less food and had therefore a lower caloric intake than did the animals fed by stomach tube. Diets devoid of threonine or histidine, respectively, caused less severe lesions than methionine deficiency. Fed for three days some of these deficient diets caused visceral lesions. M. SILBERBERG

Amino Acid Imbalance and Nitrogen Retention. P. D. Deshpande, A. E. Harper, and C. A. Elvehjem. *J. Biol. Chem.* 230: 335, 1958.

Weanling rats on a low-protein diet (6 per cent fibrin) retained about 76 per cent of their ingested nitrogen. The addition of 0.4 per cent of DL-methionine and 0.6 per cent of DL-phenylalanine created a severe imbalance resulting in both lowered food intake and decreased percentage of retained nitrogen, causing a severe depression in total retained nitrogen and rate of growth. Slight adaptation occurred over a period of six days, as was evidenced by small increases in food intake, nitrogen retention, and growth rate. The addition of 0.2 per cent of L-histidine-HCl, 0.4 per cent of L-leucine, 0.4 per cent of DL-isoleucine, and 0.6 per cent of DL-valine to the imbalanced diet resulted in a considerable increase in food intake, growth, and total nitrogen retention, but only a slight increase in the percentage of ingested nitrogen retained, as this mixture was still suboptimal in lysine content.

With a limited supply of amino acids in an imbalanced situation, the excretion of surplus amino acids appeared to be associated with an increased loss of the most limiting amino acids, as was evidenced by poorer retention of ingested nitrogen. It was suggested that rate of growth is determined by the amount of the limiting amino acid ingested, but efficiency of nitrogen utilization is determined by the relative proportions of the amino acids. There appeared to be an improvement of nitrogen utilization with lower food intake, an observation that may be important in evaluating the maintenance of humans on low-protein rations.

M. K. HORWITT

The Effect of Amino Acids on the Survival of the Hyperthyroid Rat. R. E. Boldt, A. E. Harper, and C. A. Elvehjem. *Arch. Biochem.* 74: 437, 1958.

Animals in the hyperthyroid state are known to have increased requirements for many nutrients. Weanling rats placed on a diet with 24 per cent casein and 0.4 to 0.5 per cent iodinated casein had average survival times of 21 to 28 days. Various levels of supplementation with DL-methionine up to 0.6 per cent, and betaine up to 1.0 per cent prolonged survival up to 45 days or more. The highest supplementation of DL-methionine retarded growth, but betaine had only a slight effect on weight gain. Phenylalanine, serine, and alanine exerted only slight protective action. It appeared that methionine and betaine prolonged survival time in hyperthyroid rats by furnishing labile methyl groups. Substitution of defatted pork for casein also extended survival to over 50 days. However, the protective action of pork cannot be explained by increased intake of methionine or other methyl donors, since pork and casein both furnish approximately the same amount of methionine.

M. K. HORWITT

Fat Deposition in Rat Tibia Due to Lysine Deficiency. E. L. Rohdenburg. *Proc. Soc. Exper. Biol. & Med.* 98: 835, 1958.

Weanling rats were fed a stock diet for 21 days and for five weeks subsequently diets supplemented with 0.2–1.0 per cent L-lysine monohydrochloride. The higher the supplement of lysine, the better were both the increase in weight and bone growth. Bone composition also approached the normal values more closely with increasing dose of L-lysine, while the fat content of the tibia decreased. The percentage of bone ash and organic residue was constant; but the amount of moisture varied inversely with the fat content. Thus optimum growth was related to a relatively low lipid content of the bones. M. SILBERBERG

In areas of the world where milk is not available, the dietary amino acid intake can be balanced by combinations of vegetable proteins, which, if fed singly, would be nutritionally inadequate. Supplementation of these diets with vitamins and calcium is required.

The Effect of a Supplementary Multipurpose Food on the Growth and Nutritional Status of Schoolchildren. V. Subrahmanyan, K. Joseph, T. R. Doraiswamy, M. Narayanarao, A. N. Sankaran, and M. Swaminathan. *Brit. J. Nutrition* 11: 382, 1957.

Considerable attention has recently been devoted to evolving supplementary foods from indigenous resources for improving the health of the vulnerable groups of the population. In this report a feeding experiment, carried on over a period of five months, was performed on 46 girls aged four to twelve years in an orphanage in India. Their poor vegetarian diet was supplemented with 2 oz daily of a multipurpose food composed of groundnut flour and Bengal-gram flour, fortified with vitamins and calcium phosphate. The children were paired according to height and weight, and one member of each pair was allocated, at random, to the control and experimental group. A significant increase in height, weight, red-cell count, and hemoglobin levels was noted in the subjects receiving this supplement. Eighteen children of the 23 in the experimental group improved in nutritional status, whereas none in the control group improved. On the other hand, 13 control children deteriorated in their general condition, but none of the experimental group showed these changes.

In view of the serious shortage of milk and other protective foods in certain areas of Asia, Africa, and South America, the large-scale manufacture and distribution of inexpensive supplementary foods, prepared from

readily available raw materials, should help effectively to combat malnutrition. S. O. WAIFE

NUTRITION NOTES

The influence of the composition of the diet upon the growth rates and nutritional status of experimental animals is of considerable interest. The possible clinical implications of dietary alterations, including high cellulose; carbohydrate intakes; and exercise can be drawn from the following.

Relationship Between Food Intake and Osmotic Effect of Dietary Carbohydrate. A. E. Harper and H. E. Spivey. *Am. J. Physiol.* 193: 483, 1958.

The objective of this study was to determine whether differences in the food intake and in the rate of gain of rats fed on low-protein diets containing different carbohydrates could be related to differences in the osmotic effects of the various carbohydrates. Rate of gain, food intake, stomach volume, dry weight and moisture content of the stomach contents, and the rate of stomach emptying were determined in rats at intervals after feeding low-protein diets containing various carbohydrates. There was an inverse relationship between the capacity of the dietary carbohydrate to exert osmotic pressure and the food intake and rate of gain of rats fed for a single interval each day, but the positions of glucose and sucrose in the series were reversed under conditions of ad libitum feeding. There was a direct relationship between the capacity of the dietary carbohydrate to exert osmotic pressure and the stomach volume and moisture content of the stomach contents. Rats could be trained to consume an adequate quantity of a diet in a limited time more readily if the osmotic capacity of the dietary carbohydrate was low. AUTHORS

Interactions of Dietary Fiber and Protein on Food Intake and Body Composition of Growing Rats. J. H. Meyer. *Am. J. Physiol.* 193: 488, 1958.

The addition of cellulose to the diet increases protein needs as measured by gains in the fat-free body of ad libitum and pair-fed growing rats. This was attributed to loss of metabolic fecal nitrogen induced by dietary cellulose addition. It was demonstrated that 30 per cent cellulose addition to the diet caused a need for about 1.85 per cent additional dietary crude casein. Rats fed very low-protein rations had a larger portion of their gain as fat because their intake of energy was high in relation to protein intake. This excess energy therefore limited an increase in food intake by which the animal might obtain more protein from these low-protein rations. When cellulose replaced the energy portion of the diet (sucrose), food intake increased because the animal no longer needed to dispose of the excess energy as body fat or heat. Therefore, protein in low-protein rations influences food intake indirectly through its ratio to available dietary energy. AUTHORS

A Note on the Relation of Appetite to Exercise. R. Passmore. *Lancet* 1: 29, 1958.

It is widely held by both doctors and the lay public that exercise is an unsatisfactory treatment for obesity; they argue that the extra food intake provoked will more than cancel the energy value of the exercise. Work with rats has suggested that this is not the case, and in the present paper some experience with a dog is very briefly reported. The dog, allowed to eat ad libitum once daily, consumed no more on days where it took an hour's hard exercise than on days when it was confined to its kennel. This is taken to indicate that the appetite is related to physical activity only above a minimum level of exercise and that in a sedentary existence, such as followed by the average town-dweller, the exercise is below this minimum and appetite may be out of proportion to needs. This is a fascinating idea but an unwarranted extrapolation of data from a very short-term experiment on a single dog.

F. E. HYTTEN

Numerous studies have attested to improved performance and efficiency in human subjects consuming a breakfast of adequate protein and caloric value. These findings are supported by the observations that absenteeism is higher in those with poor dietary habits.

Nutritive Intake of Women Factory Employees. R. M. Peel and M. L. Dodds. *J. Am. Dietet. A.* 33: 1150, 1957.

Two groups of women factory workers selected on the basis of their absence records cooperated in a study of dietary status. Thirty-one women in a high-absence group had an average of 23.3 days absence due to illness. The average age of this group was 48.4 years. Forty-one women who had no absence from work due to illness had an average age of 44.4 years. On the basis of a one-week dietary record the low-absence group consumed diets superior in intakes of calories, protein, calcium, iron, vitamin A, and ascorbic acid to those recorded by the high-absence group. The authors emphasize that the conditions of the study do not enable one to assign a causal relationship on the one hand between the excellence of the diet and lack of absence or on the other hand to the poor dietary status of the high-absence group and absences due to illness. Thus, the concomitant occurrence of high-absence records due to illness and dietary habits leading to inferior nutritive intake can only be pointed out.

In addition, comparisons were made with recommended (N.R.C.) dietary allowances. The high-absence group fell below these allowances for all the nutrients listed above, whereas the low-absence group met the average allowances or exceeded them except in the case of calories, calcium, and iron, though the iron intake fell only slightly below the recommendation.

When subjects were grouped according to whether they exceeded the "desirable" weight by 20 per cent or more it was found that 12 (29.2 per cent) of the non-

absence and 14 (48.1 per cent) of the high-absence fell into this category.

It would have been interesting to make a thorough physical examination of all subjects to see the extent to which physical defects may have accounted for this grouping into high- and low-absence members. In addition some supplementation of the diets of both groups with missing nutrients would have thrown some light on the question as to whether the absences due to illness may have been promoted by poor nutrient status. J. M. SMITH

The need for a higher protein intake in tuberculous patients has long been accepted. Supplementation of the diet with milk products is an excellent means for achieving the desired level of dietary protein.

Nutrition in Tuberculosis: IV. Adaptation to Hospitalization. N. L. Wilson, R. H. L. Wilson, and S. M. Farber. *J. Am. Dietet. A.* 33: 1274, 1957.

During a four-month period, all adult patients admitted to a tuberculosis hospital had a careful nutritional survey. Fifty-eight men and 18 women formed the patient group. The mean caloric intake of protein was lower for these subjects prior to hospitalization than after. There was evidence reconfirming the finding that alcoholics require more calories to gain weight than others. In addition, patients receiving para-aminosalicyclic acid (PAS) consume more food. A low income was associated with a low protein intake but not necessarily with underweight before hospitalization. Discharge against medical advice was associated with alcoholism, low income, low preadmission protein intake, and lower than average acceptance of the hospital diet.

The major increase in protein intake during hospitalization was in milk. Eggs, meat, fish, and other protein sources were little changed from the dietary habits before hospitalization. S. O. WAIFE

The Metabolism of Nitrogen, Calcium and Phosphorus in Undernourished Children. K. Joseph, M. Narayanarao, M. Swaminathan, and V. Subrahmanyan. *Brit. J. Nutrition* 11: 388, 1957.

Young girls had their inadequate vegetarian diet supplemented with 2 oz. of a multipurpose food composed of groundnut flour and Bengal-gram flour fortified with vitamins and calcium phosphate. This supplementation led to a significant increase in the retention of nitrogen, calcium, and phosphorus. This occurred in spite of an inadequate caloric intake.
S. O. WAIFE

Malnutrition Among Infants and Young Children in India. C. Gopalan. *J. Trop. Pediat.* 3: 3, 1957.

This well-documented study indicates the great extent of severe malnutrition among the young children from the poorer sections of India. According to recent census, children under the age of five constitute about 13.5 per cent of the total population of the country, but deaths in this age group account for 37 per cent of all the deaths. The expectation of life at birth is now only 32 years, compared with the 66.5 of the United Kingdom. The infant mortality rate in India is 116 per 1,000 live births, compared with 29 in the United States.

A nutritional study of 198 pregnant women in the lower socioeconomic group in one territory in India revealed that only one-third would be considered apparently healthy. Angular stomatitis and glossitis was found in 44 per cent. Hemoglobin levels of less than 10 gm./100 ml. were found in 56 per cent. Examination of nearly 1,000 infants and children under the age of five showed that the three most common signs of malnutrition were those attributable to vitamin A deficiency, protein malnutrition, and anemia. On the other hand, signs of vitamin C deficiency and rickets are relatively rare.

Milk, which would be ideal as an easily assimilable, protein-rich food is beyond the means of the poor. The solution would seem to be to increase attempts to augment and expand milk production as well as to find cheap vegetable protein foods as substitutes.
S. O. WAIFE

The mode of action of cortisone in enhancing the rate of water loss of edematous hypoproteinemic animals is not apparent. This may involve a reduction in the porosity of the capillary bed, inhibiting transcapillary loss of intravascular fluid, inhibition or reversal of secondary aldosteronism, or other metabolic effects not yet understood.

The Effect of Diuretics, Cortisone and Prednisolone on Weanling Rats with Oedema Produced by an African Plantain Diet. H. Heller, T. F. Hewer, J. M. Hughes, and H. Schneiden. *Brit. J. Nutrition* 12: 52, 1958.

A diet of boiled African plantains, deficient in protein and lipotropic substances but adequate in potassium, was fed to 232 weanling rats for three to four weeks. Arrested growth and centrilobular fatty change in the liver occurred, together with hypoproteinemia, increased total body water, and an impaired water diuresis.

Aminophylline, Diamox, Nictine, cortisone, and prednisolone increased water diuresis in protein-deficient animals, but only cortisone raised it to the level of healthy control animals. In addition, cortisone reduced the body water content of edematous rats to levels comparable with those in the control group. These findings suggest that steroid therapy is indicated in the various clinical situations in which edema and an impaired water diuresis is associated with hypoproteinemia. W. H. J. SUMMERSKILL

The Development of the Protein Fractions and of Cholesterol Concentration in the Serum of Normal Infants and Children. B. Josephson and C. Gyllensward. *Scandinav. J. Clin. & Lab. Invest* 9: 29, 1957.

Total protein, protein fractions, and cholesterol were determined in serum from 265 healthy infants and children, ranging from newborn to an age of six years. Some statistically significant differences were observed between the newborn infants and those three months old. During these first months of life albumin and γ-globulin decreased, while α-2- and β-globulin increased. During childhood α_2 was much higher than in adulthood, while γ-globulin was much lower. Cholesterol concentration was low at birth but increased slowly up to six years of age. AUTHORS

The Relationship Between Body Weight Changes and Life Duration in Male Rats. A. V. Everitt and C. Webb. *J. Gerontol.* 12: 128, 1957.

The relationship between changes in body weight and life span was studied in 68 male Wistar rats. The basic constituents of the diet, which guaranteed optimal growth, were, by weight, 21.7 per cent protein, 2.4 per cent fat, and 55.6 per cent carbohydrate. During 200 to 400 days of age, the rate of growth was significantly decreased in short-lived animals. The higher the age at which maximum weights were attained, the longer the animals lived. Inherent body size did not correlate to the life span. M. SILBERBERG

VITAMIN B_{12} AND PERNICIOUS ANEMIA

The treatment of pernicious anemia with orally administered vitamin B_{12} has been successful in most instances when a daily dose of 100 μg. or more is employed. This massive therapy appears to result in sufficient absorption of active cobalamine to promote erythropoiesis despite the low serum levels achieved by it. Parenteral treatment is considered to be more certain to produce sustained remission with greater economy.

Vitamin B_{12} in Pernicious Anaemia. Intramuscular or Oral? E. H. Hemsted and J. Mills. *Lancet* 2: 1302, 1958.

Experience in the treatment of pernicious anemia since vitamin B_{12} became available is briefly reported. The effect of varying the dosage and the route of administration was judged by changes in the red cell count. A monthly intramuscular injection of 100 μg. of vitamin B_{12} was an entirely satisfactory maintenance treatment and no advantage could be demonstrated in a higher dosage.

Over a period of two years a group of seventy-one patients taking a daily dose of 100 μg. orally an hour before breakfast were maintained as well as a group of six patients receiving the monthly injection.

F. E. HYTTEN

Absorption of Orally Administered Vitamin B_{12} in Pernicious Anaemia. J. N. M. Chalmers and N. K. Shinton. *Lancet* 2: 1298, 1958.

Although absorption of small doses of vitamin B_{12} can be blocked when used with intrinsic factor from hog stomach, it is known that the administration of large doses of vitamin B_{12} alone can maintain a permanent remission in pernicious anemia. The results of trial treatments in twenty-two cases of pernicious anemia are presented in detail.

Vitamin B_{12} alone can be absorbed and utilized by patients with pernicious anemia; the speed of response depends on the dosage but daily oral dosage of 100 μg. or more can be effective. Hematologic and clinical remission may occur without return of the serum level of vitamin B_{12} to normal.

Although daily oral treatment is effective, particularly with cooperative patients, "parenteral therapy with vitamin B_{12} is still the most reliable method of ensuring freedom from relapse." F. E. HYTTEN

Absorption of Vitamin B_{12} in Pernicious Anaemia. Defective Absorption Induced by Prolonged Oral Treatment. M. Schwartz, P. Lous and E. Meulengracht. *Lancet* 2: 1200, 1958.

It has been firmly established that while persons with pernicious anemia may be kept in permanent remission by treatment with the original relatively crude extracts of hog stomach, blockage of vitamin B_{12} often occurs when more modern purified hog pylorus is used in oral preparations with vitamin B_{12}.

In the experiments reported here absorption of vitamin B_{12} by patients with pernicious anemia was determined after dosage with a variety of intrinsic factor preparations. The early findings were confirmed: that the initial response is excellent but is not long sustained. There is no information about the duration of the blockage.

The blockage appears to be toward vitamin B_{12} bound to hog pyloric mucosa and is maintained even when human intrinsic factor is given in addition. But vitamin B_{12} given with either human intrinsic factor, or intrinsic factor from a rat in "blocked" patients is well absorbed.

The contention that the basis of the blockage is an immunity phenomenon and that intrinsic factor is species-specific is supported. F. E. HYTTEN

The phenomenon of vitamin B_{12} binding in serum and in tissues has been investigated extensively. It is of interest that bound-B_{12} is absorbed from the gastrointestinal tract more effectively than the free vitamin. It is possible that the bound material is protected from utilization by microorganisms within the gut permitting greater absorption. Similar observations with bound-B_{12} in patients with fish tapeworm infestation would be of importance.

Intestinal Absorption of Radiovitamin B_{12} Bound in Pig Liver. W. Nyberg and P. Reizenstein. *Lancet* 2: 832, 1958.

The absorption of vitamin B_{12} should be more properly studied, in physiologic terms, with naturally occurring "bound" vitamin than with the purified crystalline product.

In this experiment "natural," labeled vitamin B_{12} was prepared by feeding it to a pig for a month; its liver, containing about 6 mμg. of CO^{60}-labeled vitamin B_{12} and 560 mμg. of non-labeled vitamin B_{12} per gram of wet tissue, was fed to five healthy subjects and four patients with pernicious anemia. With an intake of 27 to 29 μg. vitamin B_{12}, an average of 32.6 per cent was absorbed by the normal subjects; in the anemic subjects an average of 61.2 per cent was absorbed from 14 to 35 μg. When crystalline vitamin B_{12} was given to these subjects in doses of from 20 to 50 μg. the mean absorption was only about 4 per cent in the normal subjects and 6 per cent in the anemic subjects. Some preliminary steps to determine the substance binding the vitamin in liver are outlined; at present it is unknown F. E. HYTTEN

Urinary Excretion of Radiovitamin B_{12} in Carriers of Diphyllobothrium Latum. W. Nyberg, R. Grasbeck, and V. Sippola. *New England J. Med.* 259: 216, 1958.

The urinary excretion of radioactive vitamin B_{12} was determined using Schilling's urinary radioactivity test in fifty non-anemic carriers of the fish tapeworm Diphyllobothrium latum, seven tapeworm carriers with megaloblastic anemia, sixteen patients with genuine pernicious anemia and fifty healthy control subjects. The excretion of the radioactive vitamin B_{12} was measured before and after worm expulsion. The worm carriers with megaloblastic anemia excreted amounts of the radioactive vitamin similar to those with pernicious anemia before expulsion of the worms. After expulsion, excretion of vitamin B_{12} returned to normal in all but two. Only nine of the fifty non-anemic tapeworm carriers showed undisputably normal excretion levels of vitamin B_{12}. The other values were low or borderline. After worm expulsion, the values increased significantly. From this data it would appear that D. latum interferes in some manner with absorption of vitamin B_{12}. The authors mention the possibility that the worm named D. latum is not the same species in different parts of the world.

M. W. BATES

Co^{60} Vitamin B_{12} Binding Capacity of Normal Human Saliva. R. W. Bertcher, L. M. Meyer, and I. F. Miller. *Proc. Soc. Exper. Biol. & Med.* 99: 513, 1958.

The vitamin B_{12} binding-capacity of human saliva was determined by incubation with Co^{60}-vitamin B_{12}, prolonged dialysis and counting the residual radioactivity: 103 normal adults gave values between 13 and 80 mμg./ml. with a mean of 31 ± 10.2 mμg./ml. There was no relationship between vitamin B_{12} binding and the specific gravity, refractive index, optical density, leukocyte count and ABO blood group-substance titer of the saliva. G. WALKER

The high concentration of vitamin B_{12} in bile compared to that of the fecal and urinary excretory routes suggests that intestinal resorption and reutilization by the liver is a mechanism involved in conservation of the vitamin. This may account for the long latent period noted before the development of pernicious anemia in patients surviving total gastrectomy.

Biliary and Fecal Vitamin B_{12} Excretion in Man. An Isotope Study. R. Grasbeck, W. Nyberg, and P. Reizenstein. *Proc. Soc. Exper. Biol. & Med.* 97: 780, 1958.

Normal subjects and patients with bile duct cannulae were given a single intramuscular injection of 0.5 μg. of Co^{56}-labeled vitamin B_{12} (activity 2 mc. per mg.), and the rate of excretion of Co^{56} in the urine, feces and bile was measured. Urinary excretion was negligible after two days. After an initial fall, the radioactivity of bile and feces remained constant during periods of observation of ten and twenty-eight days, respectively. Assuming equilibration with the body vitamin B_{12} pool by the fourth day, it was estimated that the daily output of endogenous vitamin B_{12} was 0.8 ± 0.8 μg. in the urine, 12 ± 1 μg. in the feces, and after correcting for incomplete collection, 43 ± 12 μg. in the bile. These findings demonstrate an enterohepatic circulation of vitamin B_{12} and suggest that the requirement of the vitamin in man may be greater than has hitherto been recognized.

G. WALKER

Spontaneous Remission in Pernicious Anemia. K. R. Tanaka and G. O. Clifford. *New England J. Med.* 258: 7, 1958.

Addison's pernicious anemia usually is considered irreversible although temporary remissions have been known to occur. These remissions may be due to increased production of intrinsic factor. A case is reported in this paper of a woman who was diagnosed in 1947 as having Addison's pernicious anemia and treated with liver extract or vitamin B_{12} injections. Nine years later an adequate production of intrinsic factor was shown in the patient using the Schilling test. It is unfortunate that the intrinsic factor production was not measured when the woman first came under observation to make certain the diagnosis of pernicious anemia. However, the authors in examining all the clinical data regard this diagnosis as correct, and that a remission did occur. M. W. BATES

The mechanism by which tobacco smoking reduces the serum levels of vitamin B_{12} is entirely conjectural. The effect upon intrinsic factor activity in these patients would be of interest.

Tobacco Amblyopia: A Clinical Manifestation of Vitamin B_{12} Deficiency. J. M. Heaton, A. J. A. McCormick, and A. G. Freeman. *Lancet* 1: 286, 1958.

It has been noted that tobacco amblyopia may be cured by the administration of vitamin B_{12} even if smoking continues. Fourteen subjects with the condition (which is elaborately defined) had a full blood examination, gastric analysis, liver function tests and an estimate of the serum vitamin B_{12} level. There are detailed descriptions of some of these cases and the results are presented in full.

The serum vitamin B_{12} levels ranged from 15 to 350 $\mu\mu$g./ml. (mean 218) compared to a normal mean of 538 $\mu\mu$g./ml. and it is suggested that with this degree of vitamin deficiency the retina or the optic nerve is unduly sensitive to tobacco. Other clinical manifestations of vitamin B_{12} deficiency may be absent and the serum level of the vitamin should be measured in any case of unexplained peripheral neuropathy.

In these subjects the treatment with the vitamin could reverse the changes, even if the use of tobacco continued. F. E. Hytten

Previous investigations on the influence of adrenocortical steroids upon vitamin B_{12} metabolism have indicated that the urinary excretion of the vitamin is enhanced by these hormones.

Observations on Vitamin B_{12} Absorption in Primary Pernicious Anemia During Administration of Adrenocortical Steroids. J. W. Frost and M. I. Goldwein. *New England J. Med.* 258: 1096, 1958.

A case report is presented in which vitamin B_{12} absorption was studied in a patient with pernicious anemia receiving an adrenocortical-steroid preparation. The Schilling test, which measures the urinary excretion of an orally administered dose of radioactive cobalt-labeled vitamin B_{12}, was used. The untreated patient excreted 0.2 to 0.4 per cent of the radioactive vitamin in a twenty-four-hour period. When a potent intrinsic factor was given, 14 per cent was excreted. After seven days of receiving 20 mg. of prednisone daily, excretion was 2.8 per cent and after fourteen days, 11.9 per cent. Gastric juice was obtained while the patient was receiving prednisone and given to another patient with pernicious anemia. The gastric juice contained negligible amounts of intrinsic factor since the vitamin B_{12} absorption of the second patient was not increased a significant amount. This would indicate that the increased absorption of radioactive vitamin B_{12} was not due to an increased intrinsic factor production of the patient receiving prednisone. A similar study was made on two other patients with pernicious anemia. In these two the administration of prednisone did not increase vitamin B_{12} absorption. M. W. Bates

The role of vitamin B_{12} in protein biosynthesis has been recognized as involving the formation and transfer of methyl groups as well as the synthesis of nucleic acid. Since these factors are required in the production of enzymes, it is apparent that vitamin B_{12} deficiency would evoke widespread defects in tissue metabolism.

Vitamin B_{12} and Protein Biosynthesis. IV. In Vivo and In Vitro Studies. S. R. Wagle, R. Mehte, and B. C. Johnson. *J. Biol. Chem.* 230: 137, 1958.

Uniformly labeled glucose and β-labeled serine were administered to young vitamin B_{12}-deficient rats and pigs, and the incorporation of C^{14} was followed in TCA-precipitated liver protein. Precipitates from vitamin B_{12}-deficient animals were found to contain less radioactivity than similar preparations made from adequately supplemented animals.

Isolated microsomal fractions from liver and spleen of vitamin B_{12}-deficient rats incorporated less labeled methionine, alanine and phenylalanine into protein than similar fractions from control animals. The addition of vitamin B_{12} *in vitro* greatly increased incorporation of amino acid. Microsomal incorporation of labeled phenylalanine was much greater when the incubation medium was supplemented with 19 additional amino acids than when only one amino acid was present, suggesting a requirement for an amino acid pool and that a net synthesis of protein occurred in the test system. ATP and GTP were required for maximal labeling of proteins in the presence of vitamin B_{12}. Vitamin B_{12} labeled with Co^{60} was taken up primarily by the microsomal and supernatant fractions of the homogenate.

The possible role of vitamin B_{12} in protein synthesis may be a reason why its deficiency affects a wide range of biological functions, since a lack of this vitamin could lead to a reduction of a variety of enzymes in the body. M. K. Horwitt

Results of Three Years' Experience with Microbiological Assay of Vitamin B_{12} in Serum. G. H. Spray and L. J. Witts. *Brit. M. J.* 1: 295, 1958.

This is a useful paper setting out the distribution of serum vitamin B_{12} activity in normal subjects and in subjects with a variety of diseases. The range for 123 normal subjects was 150 to 1,000 $\mu\mu$g./ml. with a mean of 450 $\mu\mu$g./ml.

Of ninety-four patients with pernicious anemia in relapse, eighty-five had levels below 100 $\mu\mu$g./ml. and all but two, who had anomalous results, were below 170 $\mu\mu$g./ml. In subacute combined degeneration of the spinal cord the vitamin B_{12} level is similar to that in patients with pernicious anemia; patients with other diseases of the nervous system have normal levels.

In pernicious anemia of pregnancy, and megaloblastic anemia associated with gastrectomy, diverticulosis of the small intestine, the malabsorption syndrome and dietary deficiency the mean level was always lower than normal but in many individuals it fell well within the normal range.

Abnormally high results were always found in patients with chronic myeloid leukemia, but results were normal in those with lymphatic leukemia. In

the acute leukemias and polycythemia vera both normal and high results were found. F. E. HYTTEN

Factors Influencing the Destruction by Heat of Vitamin B_{12} in Milk. J. E. Ford. *J. Dairy Res.* 24: 360, 1957.

This is a report of an extensive line of investigation in which many substances were added to milk and to buffered solutions of vitamin B_{12} to try to discover the factors that accelerate the destruction of this vitamin by heat, and how this destruction can be reduced.

Heat was applied to the milks and to buffered solutions of vitamin B_{12} generally at two levels—110°C. for twenty minutes and 120°C. for thirty minutes. The author reports the percentage losses of vitamin B_{12} when the following among other substances were added to milk each separately, and in various combinations: ascorbic acid, glutathione, sodium cyanide, potassium metabisulphite, sodium nitrite, and a number of other compounds that might affect the stability of vitamin B_{12}. For the most part the substances added were selected on the theory that in heat destruction of vitamin B_{12} the reaction is essentially oxidative.

De-aeration of milk before heating was observed greatly to reduce the loss of vitamin B_{12}. Ascorbic acid markedly increased the loss, as did also SH compounds. Cyanide and bisulphite reduced the loss. A buffered solution of vitamin B_{12} to which ascorbic acid was added did not show appreciable heat loss in an atmosphere of nitrogen. However, under similar conditions the addition of dehydroascorbic acid resulted in considerable loss. Both forms of ascorbic acid produced loss of vitamin B_{12} when oxygen was present.

Milk "protected" through the addition of various chemicals showed no greater reduction at the higher heat exposure than at the lower. From this the authors conclude that the destruction is not purely thermal, nor is it a simple interaction with some native constituent of the milk. The theory is advanced that in the destruction of vitamin B_{12} in milk when heated in the presence of oxygen, a causative factor may be found in the pathway of the breakdown of ascorbic acid.

The original paper should be consulted for the numerous data reported. In one directly comparative test a sample of raw milk protected from exposure to the air contained 2.9 mμg./ml. of vitamin B_{12}; after bottling and heating to 110°C. for twenty minutes—1.4; the same milk aerated then heated similarly—0.63 mμg./ml. FRANK E. RICE

INTRINSIC FACTOR

This hematopoietic factor functions to make vitamin B_{12} available for absorption through the intestinal wall or to prevent its destruction by intestinal microorganisms. There is good evidence to indicate that intrinsic factor potentiates the action of vitamin B_{12} at the tissue level, including the bone marrow, apart from aiding its absorption or preventing bacterial destruction of it. Parenterally administered vitamin B_{12} may be activated by an extragastric intrinsic factor.

Effect of Intrinsic Factor Concentrates on Vitamin B_{12} Receptor Protein of Tissues. O. N. Miller and F. M. Hunter. *Proc. Soc. Exper. Biol. & Med.* 97: 863, 1958.

The addition of an intrinsic factor concentrate increased the binding of Co^{60}-labeled vitamin B_{12} by rat serum and by homogenates of all organs tested. The binding factor in the homogenates was shown by centrifugal separation technics to be present in the soluble cell protein and the microsomes. It was not found in the nuclei, mitochondria, or cell membranes.

It is suggested that intrinsic factor is absorbed into the blood where it causes binding of vitamin B_{12} to a serum protein receptor and facilitates its entry into cells, also by stimulating protein binding.

G. WALKER

Acquired sensitivity to hog stomach mucosa may account for the gradual failure of the intrinsic factor activity of this preparation.

Intrinsic Factor-Inhibiting Substance in Serum of Orally Treated Patients with Pernicious Anaemia. M. Schwartz. *Lancet* 2: 61, 1958.

Pernicious anemia responds almost always to initial treatment with vitamin B_{12} and preparations of hog gastric mucosa, but it has been repeatedly shown that relapse occurs in a substantial proportion of patients receiving prolonged treatment. The reason for this acquired failure of treatment is a block to the absorption of vitamin B_{12} only when intrinsic factor is given as hog stomach; it is not present when homologous intrinsic factor is used.

In the experiments reported here, it is shown that sixteen of twenty-one patients treated orally for over a year had serum which actively inhibited intrinsic factor. Little is known about the factor or about its actions; it seems to operate against human intrinsic factor, and not all subjects who have it are unable to absorb vitamin B_{12}. The factor is not present in healthy persons or in patients with untreated pernicious anemia.

F. E. HYTTEN

A considerable amount of information concerning the biochemical nature of intrinsic factor has been obtained characterizing it as a mucoprotein related to blood group substrates and to a mucopolysaccharide found in colostrum.

Intrinsic Factor and Vitamin B_{12} Absorption. A. L. Latner. *Brit. M. J.* 1: 278, 1958.

Three highly purified substances with intrinsic factor activity have been prepared. They have molecular weights of 5,000, 15,000, and 40,000, and appear to be

mucoproteins. The biggest molecule may be the precursor of the others; it is the only one with much vitamin B_{12}-binding activity.

Another mucoprotein of similar molecular weight but with a different tyrosine-tryptophan ratio may occur with intrinsic factor, and is inhibitory to vitamin B_{12} absorption. This has caused some confusion which is still to be cleared up, but it may explain some of the finding of resistance in man to hog's stomach preparations which have been attributed to species specificity. This inhibitory substance may maintain its effect for a considerable period in the body.

Intrinsic factor secretion is under the influence of the parasympathetic nervous system and possibly also the thyroid.

It is likely that some form of binding to protein is necessary for the intestinal absorption of vitamin B_{12}, and there is evidence that an enzyme mechanism is involved. Absorption takes place in the middle third of the small intestine of the rat but possibly in the lower ileum in man.

Sorbitol, a hexahydric alcohol derived from glucose, increases the absorption of vitamin B_{12}, but only when the vitamin is taken in much greater than physiologic amounts. The mechanism, and the significance, of this observation is not clear. F. E. HYTTEN

Distribution of "Intrinsic Factor" Activity. V. Herbert and T. H. Spaet. *Am. J. Physiol.* 195: 194, 1958.

After preincubation with hog intrinsic factor concentrate (HIFC), marked enhancement of Co^{60}-labeled vitamin B_{12} uptake at 0°C, by rat and human liver slices, and by segments of rat small intestine, was observable. No such marked enhancement by HIFC was observable in rat kidney, heart, or spleen slices. After preincubation with serum from normal human beings or patients with pernicious anemia, slight enhancement of Co^{60}-B_{12} uptake by rat liver slices was noted; marked enhancement was noted after preincubation with a fifteenfold concentrate of normal human serum. The hypothesis is presented that there may exist a "circulating intrinsic factor" involved in the selective deposition of vitamin B_{12} in the human liver. AUTHORS

It is hazardous to draw conclusions concerning the mechanism of action of an agent derived from a different species than that employed in the investigation. In the following, hog stomach mucosa is employed to examine the action of intrinsic factor in rat liver slices. Previous reports have indicated that refractoriness to hog stomach mucosa is common in persons treated with it.

Studies of the Mechanism of the Effect of Hog Intrinsic Factor Concentrate on the Uptake of Vitamin B_{12} by Rat Liver Slices. V. Herbert. *J. Clin. Invest.* 37: 646, 1958.

The uptake of vitamin B_{12} by rat liver slices was studied. Vitamin B_{12} was labeled with $cobalt^{60}$, and a hog intrinsic factor concentrate was added. Under the conditions of this experiment there was enhancement of vitamin B_{12} uptake by the liver in the presence of intrinsic factor concentrate but only in the presence of calcium in adequate concentration. The major portion of the vitamin B_{12} (as determined from the $cobalt^{60}$) which was taken up by the liver under these conditions, may be subsequently removed by *in vitro* incubation in the presence of ethylene diaminetetraacetate (EDTA). The data suggest that the mechanism whereby intrinsic factor enhances vitamin B_{12} uptake may be physical rather than metabolic. S. O. WAIFE

Effect of Intrinsic Factor on Vitamin B_{12} Uptake by Rat Intestine In Vitro. E. W. Strauss and T. H. Wilson. *Proc. Soc. Exper. Biol. & Med.* 99: 224, 1958.

Sacs of everted rat small intestine were incubated in a saline-bicarbonate solution containing Co^{60}-vitamin B_{12} and glucose. The addition of neutralized rat gastric juice increased the uptake of Co^{60} by the preparation, the greatest effect being seen with segments from the upper ileum. Experiments in which preparations were incubated in turn in separate media containing labeled vitamin B_{12} and neutralized rat gastric juice showed that neither vitamin B_{12} nor intrinsic factor were adsorbed on to the intestinal mucosa.

G. WALKER

Different views concerning the action of sorbitol on vitamin B_{12} absorption are expressed in the following.

Enhancement of Vitamin B_{12} Absorption by D-Sorbitol as Measured by Maternal and Fetal Tissue Levels in Pregnant Rats. E. G. Rice, J. F. Herndon, E. J. Van Loon, and S. M. Greenberg. *Am. J. Physiol.* 193: 513, 1958.

Tissue vitamin B_{12} levels were determined in the blood, liver, kidneys, gastrointestinal tract and uteri and contents in pregnant and non-pregnant female rats following the oral administration of three doses of radioactive vitamin B_{12} (Co^{60}). Differences in radioactivity of tissues of animals adminstered Co^{60}-vitamin B_{12} in water and in D-sorbitol at different stages of pregnancy were noted. Administration of D-sorbitol significantly enhanced vitamin B_{12} absorption in both pregnant and non-pregnant rats. Gestation, but not D-sorbitol, causes an alteration of the tissue distribution pattern of the vitamin. As fetal growth progressed the fetuses obtained a large proportion of the absorbed B_{12} which would normally have gone to the maternal tissues. During growth of the total fetal mass from 3 to 30 gm. the amount of B_{12} retained per gram of tissue remained fairly constant. The amount of B_{12} taken up by the fetuses was to a great degree controlled by the amount of the vitamin absorbed by or stored in the mother during gestation. AUTHORS

Effect of D-Sorbitol on Absorption of Vitamin B_{12} by Pernicious Anemia Patients. L. Ellenbogen, V. Herbert, and W. L. Williams. *Proc. Soc. Exper. Biol. & Med.* 99: 257, 1958.

Five patients with pernicious anemia in relapse were given Co^{58}-labeled vitamin B_{12} orally. Absorption, assessed by measuring urinary radioactivity, was unaf-

fected by sorbitol in doses up to 10 gm. and sorbitol did not potentiate the action of ingested intrinsic factor. It had previously been shown by other workers that the administration of sorbitol increased the absorption of vitamin B_{12} in healthy adult man and rats.

G. WALKER

Pernicious Anaemia Treated Orally with "Bifacton." Refractoriness to Potent Animal Intrinsic Factor. J. B. Stokes and W. R. Pitney. *Brit. M. J.*, 1: 322, 1958.

A case is reported of a 52-year-old woman with pernicious anemia who became refractory to oral treatment with a compound of vitamin B_{12} and animal intrinsic factor.

She was able to absorb vitamin B_{12} normally when it was given with human gastric juice.

The phenomenon is apparently not uncommon and may develop quite rapidly, as it did in this case (within eight weeks). No explanation for it is offered, but it is suggested that for pernicious anemia in remission parenteral treatment with vitamin B_{12} is to be preferred.

F. E. HYTTEN

CLINICAL AND EXPERIMENTAL ARTERIOSCLEROSIS

There are few medical problems receiving more attention than that of atherosclerosis. This is a disorder of particular interest to the nutritionally-oriented clinician and research worker because of the relationships between lipid metabolism, as affected by diet and disease, and the incidence of vascular sclerosis. However, in addition to dietary factors there are other important considerations in the pathogenesis of arteriosclerosis including heredity, exercise, smoking, intravascular pressure, emotional tension, and endocrine activity. In the following summaries consideration of the etiology of the disease is limited to dietary variables and nutrition.

Diet and Cardiovascular Disease in Finland. P. Roine, M. Pekkarinen, M. J. Karvonen, and J. Kihlberg. *Lancet* 2: 173, 1958.

It has been reported that Finland has the highest incidence of deaths from coronary disease of any European country; eastern Finland is more affected than western Finland. Weighed seven-day diet surveys were made of 84 families in summer and 40 families in winter in two contrasted areas of the country.

The average fat consumption was about 100 gm. daily, similar in winter and summer, east and west. The intake of saturated fatty acids was a little higher in the East, and that of linoleic acid and other polyunsaturated acids in the West; these differences were not significant. The consumption of iodine, ascorbic acid and vitamin E, however, was considerably higher in the West. It is thought that the low iodine intake, causing hypothyroidism and thus a rise in serum cholesterol "might be partly responsible for the high serum cholesterol levels and coronary mortality in Finland in general and in east Finland in particular."

F. E. HYTTEN

Diet and Arterial Disease in a Population Sample. H. Keen and G. A. Rose. *Brit. M. J.* 1: 1508, 1958.

The diets have been compared between 24 subjects with some evidence of "atheromatous disease" and an equal number, matched for age and sex, with no symptoms suggesting atheroma.

The material was collected originally for another purpose; the dietary data were answers to questionnaires relating to a single day's diet.

The "atheromatous" group ate more than the others, the main differences being in total calories and carbohydrate in men, and saturated fat in both sexes. "The data did not provide any evidence to incriminate any deficiency of unsaturated or long-chain fatty acids as an important aetiological factor."

In view of the tiny sample involved, the ill-defined clinical difference between the groups, and the unsatisfactory nature of the dietary data, this study adds nothing to this already confused subject.

F. E. HYTTEN

The Natural History of Atherosclerosis. The Early Aortic Lesions as Seen in New Orleans in the Middle of the 20th Century. H. C. McGill, J. P. Strong, and J. C. Geer. *Am. J. Path.* 34: 209, 1958.

The aortas of 162 white and 299 Negro patients, 1 to 40 years of age and coming to necropsy at the Charity Hospital of Louisiana and through the office of the Coroner, were studied microscopically with reference to the evolution of arteriosclerosis. The patients represented the low income groups. The earliest lesions present even in young children and consisting of lipoid streaks remain stationary for many years and eventually undergo fibrosis. Between the age of 11 and 15 years, the incidence of these changes was significantly higher in Negro persons than in white persons. Toward the end of the second decade there was a sharp rise in the white population, but throughout the period of observation the Negro persons showed somewhat more involvement than the whites. Female whites were consistently least affected.

While these changes may be reversible or regress, complications such as accretion of lipid material in the plaques, hemorrhages with thrombus formation and vascularization are thought to precipitate the progressive disease. Since this stepwise development could not be reproduced in any type of experimental atherosclerosis it is questioned whether diet plays the principal part in atherogenesis. On the other hand, the rapid increase of the early lesions during puberty suggest that some hormonal factors may be involved in the production of these early lipoid changes. Furthermore, it seems that factors that initiate the early lesions differ from those that precipitate the progressive disease.

M. SILBERBERG

Civilisation, Race and Coronary Atheroma. J. B. Hannah. *Cent. Afr. J. Med.* 4: 1, 1958.

This is a study of atheroma in natives and 22 Europeans who died suddenly from all causes and who were autopsied. The cases were selected indiscriminately except that records of Africans below the age of 25 were discarded. The atheroma was judged qualitatively by eye. It is important to note that the exact age of the Africans often could not be ascertained.

The average age of the African group was 41 compared to 45 for the European. Six Europeans were under 40 years of age.

Of the 42 Africans, only one had severe coronary atheroma and he died of a typical coronary occlusion. Two others had moderate lesions and four had mild lesions. The remaining 35 showed not the slightest trace of coronary atheroma. All 22 Europeans had coronary lesions, even the youngest, aged 15. Eight of the Europeans had severe atheromatosis, and six of these died of coronary disease. This author concludes that the evidence is substantiated that in the Bantu, while not entirely immune, coronary atheromas develop much less frequently and less severely than in Europeans, and Bantus rarely die of its complications.

S. O. Waife

Comparative Severity of Atherosclerosis in Costa Rica, Guatemala, and New Orleans. C. Tejada, I. Gore, J. P. Strong, and H. C. McGill, Jr. *Circulation* 18: 92, 1958.

An analysis of the type and severity of atherosclerosis was made of 398 aortas obtained from unselected autopsy cases at a large charity hospital in Costa Rica. The results were compared with similar analyses previously reported from New Orleans and Guatemala. The authors' "Arteriosclerotic Index" revealed no significant difference in the three population groups before the age of 30. Subsequently, intimal disease developed most rapidly in those living in New Orleans. After the age of 40, atherosclerosis increased more rapidly in persons in Costa Rica than in those in Guatemala, but remained below the level of New Orleans.

These differences were analyzed in relation to reported differences in dietary habits and in serum cholesterol and lipoprotein concentrations in the same populations. The apparent correspondence between dietary fat intake, serum cholesterol and lipoprotein levels, and the degree of aortic atherosclerosis is discussed.

W. H. Abelmann

In the following studies, several factors are discussed which may play a predominant role in the increasing incidence of arteriosclerosis observed at death.

Changing Frequency of Arteriosclerosis in Mammals and Birds at the Philadelphia Zoological Garden. Review of Autopsy Records. H. L. Ratcliffe and M. T. I. Cronin. *Circulation* 18: 41, 1958.

The records of 2,499 autopsies performed on mammals and 5,900 autopsies performed on birds who died in captivity at the Philadelphia Zoological Garden from 1916 to 1955 have been analyzed for evidence of arteriosclerosis. From 1916 to 1931 the incidence of arteriosclerosis at autopsy was about 2 per cent in mammals and birds. From 1931 to 1956 the frequency of arteriosclerosis increased about tenfold in mammals and about twentyfold in birds.

Factors that may be associated with this increased incidence of arteriosclerosis are analyzed and discussed. The change in frequency began with decreasing populations and increasing age, and continued after improved nutrition had been accompanied by further increases in age. However, it has been most pronounced since 1946, when population densities were again increased and mean ages decreased to about the levels of the 1916 to 1931 period. Thus the more recent increases in the frequency may be independent of age and diets. Their association with rises in population densities lead the authors to implicate social pressures and discuss the possible role of imbalance in adrenal secretion.

W. H. Abelmann

Effect of Small Doses of Cortisone on Lipid Metabolism and Retardation of Atherosclerosis: Probable Mechanism of Action. A. Dury and L. D. Moss. *J. Gerontol.* 13: 144, 1958.

The aim of the investigation was to study the effect of cortisone on the development of atherosclerosis in cholesterol-fed rabbits with particular consideration of the distribution of lipids in tissues, and of the size of lipoproteins in blood plasma. New Zealand adult female rabbits were fed a stock diet with or without supplement of 0.5 gm. cholesterol and injected with 5 mg. cortisone acetate for 70 days and 2.5 mg. cortisone for another 30 days. Six hours prior to termination of the experiments 0.5 μc. P^{32} were injected intravenously in order to analyze the state of the phospholipids. While cortisone markedly elevated the neutral fat in the plasma and increased the amount and turnover rates of phospholipids, it produced a reversal of the amounts of S_f 0–12 and S_f 12–400 lipoprotein classes in the circulating blood. According to microscopic studies of the aortas, cortisone decreased the incidence and severity of the atherosclerotic plaques in the cholesterol-fed rabbits; it especially attenuated the formation and aggregation of foamy macrophages associated with the development of the early atheromatous lesions.

M. Silberberg

The Effects of Acute Cardiac Infarction and of Heparin Therapy on the Lipoproteins. E. M. M. Besterman. *Brit. Heart J.* 20: 21, 1958.

Serum lipoproteins were studied by paper electrophoresis in 60 patients with angina of effort, in 35 patients with acute coronary insufficiency, in 15 cases of acute myocardial infarction and in 105 cases of established myocardial infarction, and compared with 78 control serums. Pre-β lipid was increased comparably

in patients with angina and in patients with established infarcts; it was greater in patients with acute coronary insufficiency, whereas it was significantly lower following acute myocardial infarction, but rose again during recovery.

A study of the effects of heparin on lipoproteins revealed no change in lipid pattern in 10 per cent of the cases, hypermobility of the lipoproteins in 54 per cent, and reduction of the pre-β lipid without increased mobility of the β and α fractions in 36 per cent. Increased migration of the lipoproteins also resulted from *in vitro* addition of heparinized serum, heparin, or oleic acid, or from incubation at 37°C.

In 8 of the 11 heparin-resistant cases acute myocardial infarctions had occurred and no pre-β lipid was present. The refractoriness of such patients to exogenous heparin may possibly be due to previous release of endogenous heparin. W. H. ABELMANN

While hypertension and vascular sclerosis are two distinct entities, it has been demonstrated that an increased rate of deposition of lipids occur in the arterial walls in the presence of hypertension. This is shown clearly in the pulmonary vessels when elevated pressures develop in the pulmonary circulation.

Effect of Renal Hypertension on Cholesterol Atherosclerosis in the Rabbit. I. Histopathologic and Biochemical Studies. E. R. Fisher, D. L. Creed, and W. F. Baird. *Lab. Invest.* 7: 231, 1958.

Experimental evidence during the past 16 years has demonstrated a relationship between atherosclerosis and hypertension in cholesterolized rabbits and in dogs with induced renal and postural hypertension. The postulated mechanism has been one of greater penetration and deposition of lipid within aortic intima during hypertension. A morphologic approach was used by the authors to re-explore the nature of atherosclerotic lesions in rabbits with and without renal hypertension. Collateral biochemical and electrophoretic data were collected on 68 adult male and female rabbits divided into four groups. One group of 17 received Purina chow containing 2 per cent cholesterol. A second group of 30 animals with modified Page unilateral nephrectomy and cellophane enclosure of the contralateral kidney in one stage to induce hypertension, also were on a cholesterol diet. A third group of 15 rabbits with Page procedure received regular Purina chow. A fourth group were given only unilateral nephrectomy and had a cholesterolized diet. Animals were sacrificed at 74 to 77 days after operation and/or after receiving cholesterol. A variety of newer histochemical procedures were used to study the tissues.

Cholesterolized rabbits with renal hypertension showed more severe atherosclerosis of thoracic and abdominal portions of their aorta than did normotensive animals on the same diet. Hypertensive rabbits without the cholesterolized diet did not show atherosclerosis. Aortic lesions from hypertensive cholesterolized rabbits showed a marked degree of intimal fibrosis and accumulation or "polymerization" of intimal and medial acid mucopolysaccharide of the chondroitin sulphate B type, in contrast to normotensive cholesterolized animals. The authors suggest that vascular injury is associated with atherosclerosis and is increased by hypertension. There were no significant differences observed in the biochemical data. The liver was the only organ that showed significant changes in weight or lipidosis, in the hypertensive cholesterolized rabbits rather than in normotensive animals receiving cholesterol.

To what extent is aortic mucopolysaccharide active in atherosclerosis, rather than behaving as an "anatomic filler?" Does it have a heparin-like action that releases lipids from lipoproteins? Their observation of the appearance of aortic lesions at sites not usually affected under normotensive conditions in rabbits (the abominal aorta), may be indirect evidence of the intraluminal effect of hypertension. Although no causal mechanism is established, the data presented suggest a need for *in vitro* as well as *in vivo* studies of effects of increased "intraluminal turbulence." E. COHEN

Blood Pressure, Cholesterol Content of Serum and Tissues and Atherogenesis in the Rat. The Effect of Variation in Blood Pressure and the Cholesterol of Serum and Tissues and the Development of Atherosclerosis on a High Cholesterol Diet. Q. B. Deming, E. M. Mosbach, M. Berans, M. M. Daly, L. I. Abell, E. Martin, L. M. Brun, E. Halpern, and R. Kaplan. *J. Exper. Med.* 107: 581, 1958.

Male rats of the Wistar strain weighing from 100 to 200 gm. received a diet of ground Purina laboratory chow supplemented with 4 per cent cholesterol, 1 per cent choline and 0.5 per cent thiouracil. Hypertension was induced by subcutaneous implantation of one pellet of 25 mg. desoxycorticosterone acetate and a supplement of 140 mEq./L. NaCl and 35 mEq./L. KCl in the drinking water, or by constriction of the left renal artery by a silver clip two weeks subsequent to the removal of the right kidney. The high cholesterol diet increased the concentration of cholesterol, total lipids and β lipoproteins in the serum as well as the concentration of cholesterol in liver and carcass. In addition aortic changes developed in the animals comparable to those seen in atherosclerosis in man. In hypertensive animals, the development of these changes was accelerated. There was a positive correlation between the extent of the vascular lesions and the concentration of serum cholesterol, the rise of the blood pressure and the atherogenic effect of the high cholesterol diet.

M. SILBERBERG

The protection afforded by the feeding of unsaturated fatty acids in studies dealing with experimental atherosclerosis is corroborated in the following report. It is believed that the metabolic turnover and utilization of unsaturated fatty acid-cholesterol esters is more rapid and efficient than the saturated fatty acid esters.

Hypercholesteremia and Atherosclerosis Induced in Rabbits by Purified High Fat Rations Devoid of Cholesterol. G. F. Lambert, J. P. Miller, R. T. Olsen, and D. V. Frost. *Proc. Soc. Exper. Biol. & Med.* 97: 544, 1958.

Male New Zealand rabbits weighing about 1.8 kg were fed purified diets containing 20 per cent hydrogenated coconut oil or 20 per cent safflower oil for periods of 52 or 100 days. Another group of animals was fed similar diets supplemented with cholesterol in doses from 25 to 2 per cent. Blood cholesterol values were determined and the atherogenic effects of the cholesterol-free and cholesterol-containing diets were compared. Rabbits fed the diet supplemented with cholesterol showed higher plasma cholesterol levels than those fed the cholesterol-free diet. However, as far as production of atheromas is concerned, the differences between these two diets were negligible. Rabbits fed hydrogenated coconut oil without supplements of cholesterol were susceptible to hypercholesteremia and atheroma formation than those fed safflower oil without cholesterol. M. SILBERBERG

The role of vitamin D in the production of hyperlipemia and atherosclerosis is not clear in the light of our present knowledge of its actions in animal nutrition.

Influence of Some Factors on Development of Experimental Cholesterol Atherosclerosis. A. L. Myaknikov, *Circulation* 17: 99, 1958.

This article constitutes a summary of the results of several years' study of factors influencing the development of experimental atherosclerosis produced by feeding rabbits with cholesterol at the Institute of Therapy of the Academy of Medical Sciences in Moscow.

Administration of vitamin D_2 increased both the degree and rate of development of alimentary hypercholesterolemia and the intensity of aortic lipoidosis.

Ascorbic acid significantly reduced the development of hypercholesterolemia and lessened and retarded the development of aortic lipoidosis.

Phenobarbital in sedative doses was followed by a decrease in activity and blood pressure, and aortic atherosclerosis was markedly reduced.

Benzedrine resulted in greater fluctuations of blood pressure, in increased blood cholesterol, and in more extensive development of aortic lipoidosis.

While dicoumarin was without effect, heparin reduced the alimentary hypercholesterolemia as well as the aortic atherosclerosis.

Intermittent hypoxia, produced by exposure to low oxygen chambers, increased alimentary hypercholesterolemia and lipoidosis of the aorta and coronary arteries.

Physical exercise to fatigue decreased alimentary hypercholesterolemia and to some extent reduced the development of atherosclerotic changes. However, focal myocardial necrosis and fibrosis and even myocardial infarction were more frequent and more extensive than in control groups. W. H. ABELMANN

Dietary Method for Induction of Atherosclerosis, Coronary Occlusion and Myocardial Infarcts in Rats. G. F. Wilgram. *Proc. Soc. Exper. Biol. & Med.* 99: 496, 1958.

Male white rats of the Wistar strain weighing about 450 gm., fed a basal diet, served as a control group. In one experimental series the rats were kept on the basal diet supplemented by 0.3 per cent thiouracil, 0.2 per cent sodium cholate and 1 per cent cholesterol, while a third group received an additional supplement of 0.35 per cent viosterol. The observations were extended over periods up to eight months. The cholesterol-enriched diet produced hyperlipemia with mean total serum lipids of 947 mg per 100 ml. Supplements of viosterol produced coronary lesions, three types of which were distinguished histologically: (1) lipomatous plaques without signs of proliferation, present in 88 per cent of the animals, (2) atheromatous changes characterized by lipoid deposits, cellular proliferation and deposition of fibrinous material between intima and media, present in 81 per cent, and (3) atherosclerotic change showing medial calcification and lipoid deposits, marked cellular proliferation in the intima accompanied by thrombus formation and occlusion of the vascular lumen, the third group of changes were present in 33 per cent of the animals and led to myocardial infarcts in 14 per cent. The cardiac lesions were typical infarcts, different from myocardial necrosis caused by hypervitaminosis D. M. SILBERBERG

Changes in the Endothelium of the Aorta and the Behaviour of Macrophages in Experimental Atheroma of Rabbits. J. C. F. Poole and H. W. Florey. *J. Path. & Bact.* 75: 245, 1958.

Young rabbits weighing about 2 kg were fed a stock diet for four months supplemented with a solution of 5 per cent cholesterol in olive oil. The early atheromatous changes in the aorta were studied in whole thickness, "Haütchen" preparations and small fragments of endothelium detached from the surface of atherosclerotic plaques. The microscopic studies dealt specifically with the origin, development and fate of the lipid-laden macrophages appearing in close approximation to the lining endothelium of the surface. Substantial droplets of sudanophilic material were found in the cytoplasm of the endothelial cells lining the atherosclerotic plaques. These findings suggest that the function of the endothelium of aortic plaques differs from that of unchanged vascular lining cells. Possibly the lipid-laden macrophages appearing on both sides of the endothelium are formed by division of cells underneath the endothelium in which case these cells should pass through the endothelium into the blood stream; or these macrophages are derived from reticulum cells released into the blood, which attach themselves to the endothelium and pass through the latter and proliferate. M. SILBERBERG

ITEMS OF GENERAL INTEREST

Glossitis and the Pre-anaemic Stage of Pernicious Anaemia. J. F. Adams. *Lancet* 1:1120, 1957.

Three cases are described of women aged 40, 66, and 76 years who suffered primarily from soreness of the tongue. None was anemic, but vitamin B_{12} deficiency was suspected and confirmed by histamine-fast achlorhydria, early megaloblastic changes in the marrow, and a rapid response to treatment with the vitamin. The value of gastric aspiration and bone-marrow biopsy in the detection of vitamin B_{12} deficiency in nonanemic patients is emphasized. F. E. HYTTEN

Maintenance Therapy in Pernicious Anaemia: Controlled by determining Vitamin-B_{12} Level in Plasma. H. P. Østergaard Kristensen, J. Lund, A. Søeborg Ohlsen, and J. Pedersen. *Lancet* 1:1266, 1957.

The use of an oral vitamin B_{12} preparation as maintenance treatment in pernicious anemia has been evaluated in ten women aged 45 to 84 and two men 65 and 75 years, with typical pernicious anemia; the preparation used was "Cycoplex MCO" capsules containing 5 μg. of crystalline vitamin B_{12} plus intrinsic factor. Initial treatment with injected vitamin B_{12} brought the plasma level to normal, and maintenance therapy was controlled by plasma vitamin B_{12} estimations, red cell count, and marrow examinations. Even with doses of up to eight capsules of "Cycoplex MCO" daily, the serum vitamin level fell and could not be restored without parenteral treatment.

It is pointed out that treatment cannot be properly evaluated by estimation of hemoglobin and red cell levels, which may remain normal when the serum-vitamin B_{12} level has fallen and the marrow is showing megaloblastic changes. F. E. HYTTEN

Reduced Effect of Heterologous Intrinsic Factor after Prolonged Oral Treatment in Pernicious Anaemia. M. Schwartz, P. Lous, and E. Meulengracht. *Lancet* 1:751, 1957.

Treatment of pernicious anemia by a combination of oral vitamin B_{12} and intrinsic factor is widely used, but in some cases an initially good response has been followed by relapse.

A number of subjects with untreated anemia, injection-treated anemia, and orally treated anemia were investigated by means of cobalt-labeled Vitamin B_{12}. In some cases after a period of treatment with vitamin B_{12} and dried hog's stomach the absorption of the vitamin is "blocked." The block does not occur when when human intrinsic factor is used. The cause of this "block," which is presumably the reason for some therapeutic failures, is unknown. F. E. HYTTEN

A Family Study of Pernicious Anaemia. S. T. Callender and M. A. Denborough. *Brit. J. Haematol.* 3:88, 1957.

The characteristic feature of pernicious anemia is an atrophy of the gastric mucosa causing failure of the stomach to secrete intrinsic factor, but the cause of this atrophy is not yet known. It has been suggested that the tendency may be familial.

Three hundred and eight relatives of the patients (10 parents, 127 siblings, and 171 children) in 100 proved cases of pernicious anemia were investigated and compared with 259 control subjects of similar age and sex.

Methods and results are given in considerable detail. No cases of pernicious anemia were found in the controls; there were two, previously undiagnosed, among the relatives. The incidence of iron-deficiency anemia was about equal in the two groups, but macrocytosis occurred more commonly among the relatives. Achlorhydria was uncommon in both groups until the age of 40, after which it increased, more frequently in the relatives than the controls.

Some of the relatives had a low uropepsinogen excretion, gastric atrophy, poor absorption of vitamin B_{12} corrected by intrinsic factor, and a low or low-normal serum vitamin B_{12} level, but they had no anemia and were symptomless. It is suggested that they may have been in a "pre-pernicious anemia" state.

Evidence is presented to suggest that "pre-pernicious anemia" may be a carrier state, and the role of hereditary factors is briefly discussed. F. E. HYTTEN

Pituitary Growth Hormone and the Question of Pancreatic Secretion of Glucagon. O. V. Sirek, A. Sirek, and C. H. Best. *Am. J. Physiol.* 188:17, 1957.

Experiments are presented in which normal dogs were injected with a highly purified growth hormone preparation. One hour later blood samples were drawn simultaneously from the pancreaticoduodenal and jugular veins. The "central" and "peripheral" blood samples were injected into depancreatized dogs. While the injections of "peripheral" blood produced no appreciable change in the blood sugar level of the diabetic recipient, the "central" blood caused a definite but transient rise in blood sugar. The rise could be prevented by treating the recipient diabetic animal with the adrenergic blocking agent dihydroergotamine. It has been concluded that the hyperglycemic factor present in the blood of the pancreaticoduodenal vein after the injection of growth-hormone preparations is not identical with glucagon-Lilly, the effect of which is not influenced by dihydroergotamine. In two experiments in which completely depancreatized dogs, instead of intact dogs, were used as donors the administration of growth hormone produced the characteristic hyperglycemic response when "central" blood samples were injected into other depancreatized dogs. These findings cast doubt on the pancreatic origin of the hyperglycemic material and add interest to the search for its source and nature. Further experiments using depancreatized dogs are in progress.

AUTHORS

Adrenocortical Function of Hypercalcaemia of Infancy. J. Anderson, E. G. Brewis, and W. Taylor. *Arch. Dis. Childhood* 32: 114, 1957.

The urinary excretion of adrenocortical steroids before and after the administration of ACTH has been studied in two infants, one with a severe hypercalcemic syndrome and the other with the benign type of hypercalcemia. The same tests were performed on six normal children and on one child who had recovered from hypercalcemia. Total 17-ketosteroid excretion per twenty-four hours was measured in addition to plasma, calcium, sodium, potassium, and blood urea. There was little or no adrenocortical response to the administration of ACTH (1 unit every four hours for ten days) in the patient with severe hypercalcemia, and there was no significant change in the plasma concentrations of calcium, phosphorus, and urea, although there was a slight decrease in urinary calcium. This patient was later given cortisone beginning with 2 mg per day and increasing to 28 mg daily. Plasma concentrations of sodium, potassium, and cholesterol did not change whereas those of calcium, phosphorus, and urinary calcium were markedly reduced. The patient's weight gradually increased. The excretion of urinary total 17-ketosteroids gradually increased to the level found in normal infants. The pigmentation of the skin decreased considerably during the administration of cortisone.

In contrast, the patient with benign hypercalcemia responded well to the ACTH test as evidenced by a significant rise in total 17-ketosteroid excretion and a dramatic fall in plasma calcium concentration. There was also a striking fall in the plasma cholesterol level.

It is concluded that impaired adrenal function may be an important factor in hypercalcemia of infancy. Evidence presented suggests that the infant with severe hypercalcemia had primary adrenal dysfunction while the infant with the benign type had secondary adrenal dysfunction. T. C. PANOS

Use of Iodine^{131} Labeled Oleic Acid in Study of Gastrointestinal Function. J. K. Isley, Jr., A. P. Sanders, G. J. Baylin, K. W. Sharpe, J. C. Hymans, J. M. Ruffin, W. W. Shingleton, and J. R. Wilson, Jr. *Proc. Soc. Exper. Biol. & Med.* 94: 807, 1957.

Blood level activity was measured following the ingestion of I^{131} labeled oleic acid. Following a pancreatectomy the blood levels obtained with labeled oleic acid were within normal range. Animals receiving labeled triolein showed subnormal blood levels. The authors concluded that this provides means for differentiating between abnormalities of digestion and growth. L KINSELL

Significance of Waterbrash in Dyspepsia. F. Lees and F. D. Rosenthal. *Lancet* 2: 1032, 1957.

The symptom of waterbrash, quite unmistakable to those who have it, is seldom the cause of complaint and must usually be elicited by direct questioning. In this small study it was found to be commonly associated with duodenal ulcer and presumptic peptic ulcer (about 42 per cent) but much less commonly with functional dyspepsia (9 per cent).

F. E. HYTTEN

A Review on the Effects of Different Carbohydrates on Vitamin and Amino Acid Requirements. A. E. Harper and C. A. Elvehjem. *J. Agric. & Food Chem.* 5: 754, 1957.

Numerous reports in the literature are cited that demonstrate a lesser dietary need for the various vitamins contained in the B complex (mainly those other than thiamine) when an animal's diet contains carbohydrates that are less soluble, such as the starches and dextrins, as compared with diets containing soluble sugars. It has been demonstrated that this protective effect is due to a stimulated growth of intestinal microflora, capable of synthesizing many of the B vitamins. Various reports are cited indicating that while the starches and dextrins are more vitamin B complex sparing than sucrose and glucose, lactose holds an intermediate position. Later studies with the individual vitamins contained in vitamin B complex indicate that for the most part intestinal bacterial synthesis of the various members of vitamin B complex are influenced generally in the same directions by the type of carbohydrate, although there are exceptions. With respect to protein and amino acid requirements, a number of researchers have reported an apparent sparing effect from the dextrin and starch type diets as against the more soluble carbohydrates. The authors present evidence that this is not likely due to intestinal bacterial synthesis of protein or amino acids. Rather the effect may be due to the slower absorption of carbohydrate from the dextrin and starch type diet, which makes possible a more nearly simultaneous absorption of glucose with the essential amino acids. This situation should reduce the extent to which amino acids are destroyed by deamination while serving an energy-producing function. FRANK E. RICE

Effects of Epinephrine on Carbohydrate Metabolism in Underfed and Ad Libitum-Fed Rats. B. N. Spirtos, R. G. Stuelke, and N. S. Halmi. *Am. J. Physiol.* 190: 239, 1957.

Rats fed 10 gm. of a commercial diet for four to five weeks and fasted for twenty-four hours showed less rise in liver glycogen and blood sugar levels in response to the injection of epinephrine than did fasted rats fed ad libitum. Gastrocnemius glycogen levels were found to be higher in underfed fasted animals, and fell to the same extent as in fasted animals fed ad libitum, when epinephrine was given. Blood lactate concentrations, however, rose less markedly in the underfed fasted animals. This may have been at least partly responsible for the diminished rise in hepatic glycogen and blood sugar. AUTHORS

Cobalt and Vitamin B_{12} in Ruminant Nutrition: A Review. S. E. Smith and J. K. Loosli. *J. Dairy Sci.* 40: 1215, 1957.

Vitamin B_{12} is synthesized in large amounts in the stomachs of cobalt-fed ruminants, where little or none is found in the rumen contents of animals on a deficient cobalt intake. Here the production of vitamin B_{12} is bacterial. It has been found that a cobalt deficiency develops in ruminants on pastures which are adequate for horses, rabbits, and guinea pigs. There is some indication that ruminants have a greater requirement for cobalt and vitamin B_{12} than other animals. The authors believe this has not been entirely proved. Cobalt-deficient lambs have been found to respond to vitamin B_{12} therapy. It is obvious that vitamin B_{12} is one important intermediary in cobalt metabolism. Whether there are others remains to be determined.

Rumen contents of cobalt-fed ruminants are one of the richest natural sources of vitamin B_{12}. Cow's milk is an important carrier; average given for market milk is 3.2 μg. per liter. Human milk is reported to average 0.4 μg. per liter. Cow colostrum contains much more than mature milk. Pasteurization does not reduce the amount present in milk. No accurate determination has been made of the normal human requirement for the vitamin, however, to relieve pernicious anemia an accepted figure is 1 μg. per day when administered parenterally. From this the authors calculate a human requirement of 0.007 per pound of body weight per day. This is considerably less than has been determined to be the apparent requirement of many animals, when vitamin B_{12} is administered parenterally. FRANK E. RICE

The Renal Lesions in Experimental Deficiency of Potassium. W. N. Tauxe, K. G. Wakim, and A. H. Bagenstoss. *Am. J. Clin. Path.* 28: 221, 1957.

In severe hypokalemia the renal epithelia, in particular of the proximal tubules, undergo hydropic vacuolation. These vacuoles coalesce, but do not contain fat. Similar lesions could be produced in laboratory animals. Weanling rats of the Sprague-Dawley strain were fed: (1) Fuhrman's synthetic potassium-deficient diet; (2) Fuhrman's diet supplemented with potassium chloride; and (3) a commercial stock diet. Some groups were fed the deficient diets for two weeks and then received supplements of potassium for one, two, or three weeks; others were fed the deficient diet throughout the experimental period. In one set of experiments, unilateral nephrectomy was carried out after two weeks, in another no operation was performed. The potassium-deficient animals showed grossly enlarged kidneys; microscopically, the tubules were dilated, filled with calcium, and the epithelia were markedly vacuolated; some foci of necrosis in the parenchyma were seen, and some glomeruli were fibrosed. The lesions progressed in severity from the glomeruli to the distal collecting tubules. Most changes were reversible, and after three weeks restoration to normalcy had occurred except for the calcium deposits and the hyalinization of the glomeruli. M. SILBERBERG

Choline Deficiency in the Guinea Pig. R. J. Young and C. C. Lucas. *Canad. J. Biochem. & Physiol.* 35: 1, 1957.

Newborn guinea pigs fed a diet deficient in choline showed growth retardation and a 100 per cent mortality within three weeks. The livers only occasionally contained traces of stainable fat. Three- to four-week old guinea pigs fed the deficient diet for six weeks survived and showed a measurable increase in total liver fat, while the total body fat stores were being depleted. M. SILBERBERG

The Acute Renal Lesions Produced by Choline Deficiency in the Male Weanling Rat. H. C. Moore. *J. Path. & Bact.* 74: 171, 1957.

Weanling albino rats of the Wistar strain weighing about 20 gm. were housed singly and fed 10 gm. of a synthetic diet deficient in choline. Control animals were fed the same diet supplemented with 0.35 or 0.5 gm./100 ml. choline chloride. The animals were observed for periods up to fourteen days. Biopsies of kidneys were made after about seven days and the microscopic findings were compared with those found at the end of the experiment. Three types of renal lesions were noted: (1) fine fatty droplets in the loop of the proximal tubules without swelling of the epithelia. These changes were present as early as after two days and did not materially increase thereafter. The control animals showed the same changes, although less often; (2) necrosis of proximal tubules present after seven days with or without attempted epithelial regeneration; (3) cortical infarct-like necrosis with involvement of glomeruli and distal convoluted tubules present likewise after seven days. These changes were not accompanied by regeneration, or they showed early or advanced repair. The severe damage overshadowed the fatty change, and there was no correlation between the stationary fatty change and the necrotic lesions. The renal changes were thought to be due to vasospasms, and the fatty change to an over-all but non-specific deficiency.

M. SILBERBERG

An Increased Vitamin B_6 Requirement in the Rat on a Diet Containing L-Penicillamine. E. J. Kuchinskas and V. du Vigneaud. *Arch. Biochem.* 66: 1, 1957.

Previous work has shown that the addition of 0.35 per cent L-penicillamine hydrochloride hydrate to an otherwise adequate basal diet containing 1 mg. of pyridoxine hydrochloride per 100 gm. diet resulted in an immediate loss of appetite and weight, followed by acrodynia-like skin symptoms and convulsions in the male rat, similar to vitamin B deficiency symptoms. In the present studies, subcutaneous injections of 1 mg. of pyridoxine hydrochloride resulted in increased food

intake and weight gain for more than two days in rats given the same diet. Diets containing 10 mg. of pyridoxine hydrochloride per 100 gm. completely prevented or reversed the loss of appetite and weight in the presence of 0.35 per cent L-penicillamine. Increasing the other water-soluble vitamins except pyridoxine did not reverse the weight loss. The effect of inclusion of L-penicillamine can be reversed with all three isomers of vitamin B_6 (pyridoxine, pyridoxamine, and pyridoxal).

High xanthurenic acid excretion following a test dose of L-typtophan which resulted from feeding L-penicillamine was also prevented by high dietary vitamin B_6. The symptoms resulting from dietary L-penicillamine appear to be related to an increased metabolic need for vitamin B_6, with some suggestion that tryptophan metabolism is impaired. The rapidity of onset of vitamin B_6 deficiency by L-penicillamine, which produced a weight loss in twenty-four hours, and spectacularly rapid reversal by vitamin B_6, suggests a close relationship of penicillamine with a metabolically active form of pyridoxine. The possibility that the coenzyme of kynureninase might be blocked was suggested. M. K. HORWITT

Increase of Vitamin B_{12} Content of Livers of Pantothenic Acid-Deficient Rats. R. Radhakrishnamurty and P. S. Sarma. *Arch. Biochem.* 67: 280, 1957.

Previous reports have indicated that vitamin B_{12} deficiency in rats and chicks resulted in severalfold increases in liver pantothenic acid and coenzyme A. Further support of this relationship comes from the observation that hyperthyroid stimulated vitamin B_{12} deficiency had produced a fourfold increase in coenzyme A. In the present study, young albino rats, which were made deficient in pantothenic acid over a period of five weeks, showed vitamin B_{12} concentrations in liver which were about twice those in control animals. The riboflavin content of the liver was unaffected by pantothenic acid deficiency. This and the other studies suggest a reciprocal relationship between pantothenic acid and vitamin B_{12}. M. K. HORWITT

Pantothenol and the Burning Feet Syndrome. S. W. Bibile, N. D. W. Lionel, R. Dunuwille, and G. Perera. *Brit. J. Nutrition* 11: 434, 1957.

An unusual syndrome in malnutrition consists of burning and aching of the extremities. This form of peripheral neuropathy consists chiefly of symptoms rather than objective neurologic signs. It had been reported that calcium pantothenate was successful in treatment of this condition.

A controlled clinical trial on the efficacy of pantothenol was carried out in a rural area in Ceylon. Sixty patients were allocated at random into three treatment groups. One group received 500 mg pantothenol twice a week by injection, another group received it orally, and a third group received propylene glycol orally or by injection instead of the vitamin. This was a double blind test. The results did not show any significant difference in the cures obtained between control subjects and treated patients. The evidence indicates that pantothenic acid deficiency was not the cause of burning feet syndrome in the patients investigated. This is a good example of the proper approach to clinical investigation in the field of nutrition. S. O. WAIFE

Xanthurenic Acid and N_1-Methylnicotinamide in Pyridoxine Deficiency Due to Diet or to Desoxypyridoxine. L. Mainardi. *Acta Vitaminologica* 2: 73, 1957.

Pyridoxine-deficient rats (due to diet or desoxypyridoxine) have a high xanthuremia and reduced urinary elimination of N_1-methylnicotinamide. Administration of a dose of pyridoxine capable of returning to near normal the xanthuremia in rats which are pyridoxine-deficient due to diet or to desoxypyridoxine, also normalizes the reduced elimination of N_1-methylnicotinamide in deficiency caused by the antivitamin, but not in that due to the diet. AUTHOR

On the Nature of Enzymatic Defect in Phenylpyruvic Oligophrenia. C. Mitoma, R. M. Auld, and S. Udenfriend. *Proc. Soc. Exper. Biol. & Med.* 94: 634, 1957.

Patients with phenylpyruvic oligophrenia are essentially devoid of phenylalanine hydroxylase activity (in the conversion of phenylalanine to tyrosine). Phenylalanine hydroxylase consists of two protein fractions. Fraction I is present only in the liver. Fraction II is present in almost all tissues including the brain. Liver samples from normal and phenylketonuric patients were obtained at autopsy four hours after death, and kept in a frozen state until used. When rat fraction I was incubated with fraction II from phenylketonuric liver, tyrosine was formed to the same degree as when incubated with fraction II from normal liver. Fraction I activity could not be demonstrated in normal or phenylpyruvic livers. Lack of activity in normal livers was presumably due to the method of collection. Another study reporting the absence of fraction I activity from a biopsy specimen of phenylpyruvic liver would indicate that fraction I is the enzyme primarily concerned with the hydroxylation reaction. L. KINSELL

Comparison of Cardiac and Pyloric Sphincters. A Manometric Study. M. Atkinson, D. A. W. Edwards, A. J. Honour, and E. N. Rowlands. *Lancet* 2: 918, 1957.

The relation between structure and function at the esophagogastric junction and at the pylorus presents an intriguing paradox. There appears to be no anatomic sphincter at the cardia and yet it functions as a sphincter, whereas the pylorus which has all the anatomic hallmarks of a sphincter does not seem to behave like one.

In this careful study observations have been made of

pressures exerted on small rubber balloons, under a variety of conditions, at the two sites. There is a convincing demonstration of a physiologic sphincter at the lower end of the esophagus, which relaxes as an integrated part of the swallowing reflex. The action extends superiorly to the diaphragm and is clearly not due to diaphragmatic action; nor is the valve-like action of the esophagogastric junction due to a flap or funnel valve mechanism. There is an innate, active sphincteric mechanism.

In contrast, the pylorus shows no evidence of sphincteric action in normal subjects either fasting or with food in the stomach, or in patients with duodenal ulcer or in one patient with hypertrophic pyloric stenosis. "Since there does not appear to be a tonically contracted sphincter closing off the pyloric lumen, it is possible that the rate of gastric emptying is regulated by a combined filtration and pumping mechanism in this region." F. E. HYTTEN

Organic Fertilisers as a Source of Salmonella Infection. J. H. C. Walker. *Lancet* 2: 283, 1957.

In spite of great emphasis on cleanliness in the processing and handling of foods, outbreaks of salmonella infection seem to be becoming more common each year in Britain.

In the present enquiry 123 samples of a wide variety of commonly used organic fertilizers, from both human and animal sources, were examined as sources of the organisms. Fifty (40 per cent) were positive for salmonellae.

Bone meal was the most heavily contaminated (70 per cent of the specimens) and 28 of the total of 34 different salmonella types were found in it.

It is considered that some of the unexplained salmonella outbreaks in both man and animals may be due to contamination, particularly of vegetables, from fertilizers. Since many of both the raw materials and the finished products are imported their use "may be responsible for the introduction into this country and the spread among animals, of other diseases."

F. E. HYTTEN

The Metabolism of Natural and Artificial Fluoridated Waters. M. J. Wagner and J. C. Muhler. *J. Dent. Res.* 36: 552, 1957.

The fluoride metabolism of four groups of weanling rats was studied over a 40-day period. The first group of rats received drinking water from Decatur, Indiana, that naturally contained a concentration of 1.11 p.p.m. fluoride. The second group of rats, which were littermates paired with those of the first group received redistilled fluoride-free water to which enough sodium fluoride was added to give a concentration of 1.09 p.p.m. fluoride. The third group of rats received drinking water from Bartlett, Texas, where the water naturally contained 8.0 p.p.m. fluoride. The fourth group of rats, which were littermates matched to those of the third group, received redistilled water to which enough fluoride had been added to achieve a fluoride concentration of 8.0 p.p.m.

The authors concluded that there was no significant difference in skeletal retention of fluorides by rats in dependence upon whether the fluoride was added in the laboratory or in the natural watersheds. In all cases, however, the rats that received water containing naturally provided fluorides excreted slightly higher percentages of the ingested fluoride than did the rats provided the drinking solution with added fluorides. These differences were more striking among rats receiving the 8 p.p.m. fluoride waters than than those receiving the 1.1 p.p.m. waters. The authors ascribe the difference to interference with the absorption of fluoride by the inorganic constituents in the natural water supplies. Thereby they point out an error in the design of these experiments in that essentially ion-free redistilled water was used for the preparation of the added-fluoride waters for groups 2 and 4, when obviously a much fairer comparison would have been obtained if they had used water for these groups that had approximately the same ion concentration as the water from Decatur and Bartlett. J. H. SHAW

A Hormonally Controlled Serum Factor Which Stimulates Sulfate Incorporation by Cartilage In Vitro. W. D. Salmon, Jr. and W. H. Daughaday. *J. Lab. & Clin. Med.* 49: 825, 1957.

The authors present studies on a substance from serum of normal rats which promotes sulfate uptake *in vitro* by cartilage of hypophysectomized rats. They have termed this substance, the sulfation factor. The technic used was to measure the incorporation of S^{35} sulfate by cartilage *in vitro*. Both *in vivo* and *in vitro* cartilage has been shown to incorporate S^{35} sulfate in chondroitin sulfate.

The studies reported show that the incorporation *in vitro* of sulfate by cartilage from rats is reduced by hypophysectomy and can be restored by growth hormone administered *in vivo*. Insulin was also shown to stimulate the incorporation of sulfate by cartilage. Glutamine was shown not to be the serum factor which was responsible for the incorporation of sulfate. Further studies will be awaited with much interest.

K. R. CRISPELL

Enlargement of Submandibular Salivary Glands of Rats Restricted to Acidic and Basic Solutions as the Sole Source of Drinking Fluid. G. J. Cox, W. G. Clements, E. C. Hilger, Jr., and D. W. Degrange. *J. Dent. Res.* 36: 576, 1957.

Weanling rats were fed a stock ration and provided *ad libitum* over a four-week period with water or a solution containing a 0.05 molar concentration of any one of the following chemicals: sodium bicarbonate, acetic acid, lactic acid, citric acid, tartaric acid, phosphoric acid, or oxalic acid. At the end of the experiments, the rats were weighed to the nearest gram, and the submandibular glands dissected and weighed to the

nearest 0.2 mg. The ratios of the mean weight of each pair of glands to the body weight were calculated. Significant enlargements of the submandibular glands were noticed when the sole drinking fluid was unbuffered 0.05 molar acetic, lactic, citric, tartaric, or phosphoric acid as compared to the glands from the rats consuming water. The acetic acid solution caused the least enlargement while the phosphoric acid solution caused the greatest. No increase in size was noted among those rats offered the 0.05 molar solution of sodium bicarbonate or oxalic acid.

These are interesting observations and need to be extended to include acidic foodstuffs of various kinds: fruit juices and soft drinks. In addition it would be valuable to know whether salts of these acids also caused comparable enlargements of the submandibular glands. J. H. SHAW

The Treatment of Ulcerative Colitis. J. B. Kirsner, R. O. Bicks, and W. L. Palmer. *Arch. Int. Med.* 99: 642, 1957.

In this good review of the medical treatment of ulcerative colitis, the authors conclude that medical therapy is satisfactory in approximately 85 per cent of patients. Treatment must be maintained on many fronts, and consist of physical and emotional rest, the administration of sedatives, antispasmodics, and, occasionally, tranquilizing drugs.

A low-residue nutritious diet supplemented with vitamins is recommended. Correction of anemia and hypoproteinemia, as well as maintenance of electrolyte and fluid balance, is essential. Both chemotherapy and superficial psychotherapy play a role. Corticotropin and adrenocorticosteroids, although not curative, serve as "therapeutic bridges," permitting more effective application of other means of therapy. Although remission may be obtained in 85 per cent of patients, recurrences are frequent.

The acutely ill patient receives a low-residue diet containing 1,200 calories, 40 gm. protein, 60 gm. fat, and 130 gm. carbohydrate. It consists of cereal, boiled rice, poached egg, custard, and the like. Use of powdered foods rich in protein and carbohydrate is encouraged, and a more liberal intake of food is permitted as the patient's condition improves. Avoidance of irritants is very useful in relieving abdominal discomfort and diarrhea. Tube feeding may be helpful, although it is less preferable than good quality whole foods. One such preparation consists of a quart each of milk and cream, plus a vitamin-mineral-protein supplement, powdered skim milk, sugar, and homogenized and diluted lean meat. This material is introduced through an indwelling plastic catheter. S. O. WAIFE

The Physiology of Intestinal Absorption. B. I. Korelitz and H. D. Janowitz. *J. Mt. Sinai Hosp., New York* 24: 181, 1957.

In this timely review, the present knowledge of the absorption of fat, carbohydrate, protein, water, electrolytes, and iron is critically examined.

The absorption of neutral fat must be preceded by hydrolysis in the intestinal lumen for which bile salts and pancreatic lipase are essential with the formation of monoglycerides, fatty acids, and glycerol. These moities are actively transported across the intestinal mucosal cell. The long-chain fatty acids appear in the mesenteric lymph as triglycerides, while the short-chain fatty acids and glycerol enter the portal vein in their original form.

Carbohydrates are altered by digestive processes to monosaccharides prior to absorption. They cross the intestinal membrane more in the proximal than in the distal small intestine. Since the presence of carbohydrate solutions in the small intestine delays gastric emptying and, if hypertonic, promotes the passage of extracellular fluid into the intestinal lumen, such carbohydrate solutions are usually rendered isotonic in the intestinal lumen prior to absorption. Glucose, galactose, and fructose are selectively absorbed while other monosaccharides are passively translocated. The role of phosphorylation is not yet clear.

Protein is absorbed predominantly as amino acids, the L-isomer being referentially absorbed.

Water is passively absorbed, the rate being dependent on electrolyte transfer and the selective absorption of organic solutes. Electrolytes are actively absorbed. The mechanism permitting the passage of electrolytes from the blood into the intestinal lumen is obscure.

As the authors point out, there is need for more basic studies of the pathologic physiology of absorption since this may further clarify normal physiologic mechanisms. J. B. HAMMOND

Sensitization by K- or Mg-Deficiency for the Production of Myocardial Necroses by Papain. H. Selye and E. Bajusz. *Ztschr. Vitamin-Hormon-Ferm. forsch.* 10: 39, 1959.

In rats kept either on an Mg- or on a K-deficient diet for one week, the production of myocardial necroses by the intravenous injection of papain extract is greatly aggravated. This sensitizing effect of the K-deficient diet can be abolished by supplements of $MgCl_2$, and that of the Mg-deficient diet, by KCl.

AUTHORS

The Definition and Diagnosis of Hypothyroidism. P. Starr. *J. Am. Geriatrics Soc.* 7: 105, 1959.

A discussion of hypothyroidism is presented in which the author offers some very speculative ideas, assigning various thyroid hormones different functions. Included in this discussion is a report of the serum protein-bound iodine levels in some 4,500 males in California industries. In 7 per cent of these protein-bound iodine levels were less than 4 μg. per 100 ml. In a small sample of the group with low protein-bound iodine levels studied more carefully, three-quarters were thought to have hypothyroidism. These data suggest that 5 per cent of the 19 million males employed in industry have hypothyroidism. G. HOLLIFIELD